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THE BRITISH JOURNAL OF EXPERIMENTAL PATHOLOGY

VOL. XXXVII APRIL, 1956 NO. 2

RIFT VALLEY FEVER VIRUS IN MICE. I. GENERAL FEATURES OF THE INFECTION

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From the East African Virus Research Institute, Entebbe, Uganda

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RIFT Valley Fever (R.V.F.) virus was first isolated during an epizootic involving sheep and cattle in the Rift Valley, Kenya, in 1931 (Daubney and Hudson, 1931). The virus was shown to kill lambs and mice within a day or two, producing massive hepatic necrosis of which the histological picture (Daubney and Hudson, 1931; Findlay, 1933) was similar to that of yellow fever. The host range and pathology was studied, and infection in man recorded (Findlay, 1931). Subsequent workers described the neuro-adaptation of the virus (Mackenzie and Findlay, 1936; Smithburn, 1949; Kitchen, 1950), its growth in tissue culture and embryonated eggs (Mackenzie, 1933; Saddington, 1934), its oncolytic action (Takemori, 1954), and its transmission by, and isolation from, mosquitoes of the genus *Eretmapodites* (Smithburn, Haddow and Gillett, 1948; Smithburn, Haddow and Lumsden, 1949). More recently another outbreak in cattle in South Africa was reported (Gear, de Meillon, Measroch and Davis, 1951), where infections in man also occurred (Joubert, Ferguson and Gear, 1951; Mundel and Gear, 1951).

Findlay and MacCallum (1937) drew attention to similarities between R.V.F. and yellow fever viruses. Interference, but not cross-immunity (Findlay, 1931), has been demonstrated, and both viruses have been neuro-adapted and are closely similar in host range, pathology, size and clinical picture in man. R.V.F. virus however, unlike yellow fever, is viscerotropic in mice when inoculated by the intracerebral or any other route, and is so rapidly and regularly lethal that infectivity titrations are remarkably clear-cut and reproducible. This virus-host system was considered worthy of investigation because a yellow fever-like viscerotropic virus can be studied in a highly and uniformly susceptible yet comparatively cheap and plentiful laboratory animal. The system is also suited to the quantitative studies described in later papers.

MATERIALS AND METHODS

Virus.—A strain isolated in mice from pooled *Eretmapodites* mosquitoes in Bwamba County, Western Uganda (Smithburn *et al.*, 1948) was used, which had had about 90 mouse

passages. Seed virus for experiments was obtained in the form of pooled serum from sick or newly dead mice. Brains were not harvested, for not only is the titre in brain lower than in blood, but neurotropic strains of virus may thus be selected (Smithburn, 1949).

Mice.—The mice used were of Swiss stock, their ancestors originating from Carworth Farms, New York. Unless otherwise stated, mice were used when 4–6 weeks old and inoculation was intracerebral.

Infectivity titrations

Serial $10^{0.5}$ fold (or sometimes tenfold) dilutions of infective material were made and each dilution inoculated (0·03 ml.) intracerebrally to 5 or 6 mice, which were not as a rule randomised. The diluting medium was 0·5 per cent bovine plasma albumin in pH 7·4 Sørensen's buffer. Mice were observed for 5 days, and end-points then calculated from deaths by the method of Reed and Muench (1938). Deaths which occurred on the first day were not included in calculations. Organs and carcasses were made up as 10 per cent suspensions in standard diluent and the suspension considered as a 10^{-1} dilution in titrations, so that an organ titre is expressed in LD_{50} per 0·03 ml. of solid tissue. All titres are expressed in log 10 LD_{50}/0·03 ml. unless otherwise stated. Antibiotics, when used, were added so that each ml. of inoculum contained 1000 units of soluble penicillin and 1 mg. streptomycin. It was found to be important not to add too much antibiotic to the inoculum, because mice given as much as a few hundred units of penicillin or several tenths of a milligram of streptomycin alone in saline intracerebrally, died within 24 hr. This was the routine titration method used during the work described in this and the three subsequent papers of this series.

Neutralisation tests

Neutralisation tests were done intraperitoneally in adult mice, and from 10^2–10^3 intraperitoneal LD_{50} were used. Sera, including the normal mouse serum added to virus in the control titration, were inactivated for 30 min. at 56° before testing. Serum-virus mixtures remained at room temperature for 15 min. before inoculation.

Virus storage.—A fresh infective serum pool was titrated, and stored in aliquots in rubber-stoppered test-tubes at − 20°. At intervals a tube was removed and the serum titrated. As shown in Table I, the titre remained steady for at least a month ; small amounts of virus were still present after eight months. Such stock serum was used for experiments as a virus seed of known titre.

TABLE I.—*Stability of Rift Valley Fever Virus at* −20°.

	Days.						
	0.	1.	3.	5.	10.	15.	33.	69.
Titre	7·8	7·9	7·7	7·7	7·4	8·0	7·4	5·0

RESULTS

Age of mice, inoculation route and susceptibility

A given stock infective serum was titrated intraperitoneally, intracerebrally and subcutaneously in 4-day, 4-week, and 8-week-old mice. There was (Table II)

TABLE II.—*The Titre of a Given Infective Serum according to the Age of the Mice used in the Titration and the Route by which they were Inoculated.*

	Mouse age.		
	4 days.	4 weeks.	8 weeks.
Intracerebral	8·5	8·3	8·3
Intraperitoneal	8·2	7·5	7·4
Subcutaneous	8·0	7·3	7·1

Titres for 4-day mice, which received only 0·01 ml., have been multiplied by three.

some fall in susceptibility to extraneural inoculation in older mice, but the intracerebral route gave titres approximately independent of age. The result was confirmed in a subsequent experiment.

Another stock serum was titrated intracerebrally and intravenously in 5-week-old mice (6 mice per 0·5 $\log_{10}$ dilution) and the end-points found to be $10^{8\cdot8}$ and $10^{8\cdot9}$ respectively. Thus, adult mice are equally susceptible to intravenously and to intracerebrally injected virus, although they show a diminished susceptibility to intraperitoneal and subcutaneous injection. Moreover, when 10 mice were given 10 LD_{50} intracerebrally, and another 10 mice given the same inoculum intravenously, incubation periods in the two groups were identical. In another experiment, a very small virus inoculum was given intravenously to some mice, and the identical inoculum intracerebrally to others. The difference between blood and brain titres of individual mice which died was virtually the same (Table III) whether the inoculum had been given by the intravenous or the intracerebral route. There was thus no more virus present in the brain after an intracerebral inoculation. Calculations from the blood content of mouse brain as determined by Kaliss and Pressman (1950) show that the virus content of the brain can approximately be accounted for by the contained blood. Thus there is no evidence that viscerotropic virus grows in the brain to any appreciable extent, except in the exceptional circumstances described in Paper IV of this series. It is known (Cairns, 1950) that an intracerebral inoculation, apart from the inevitable damage to the brain, is in effect the same as an intravenous one, and inoculated material has begun to spill over into the neck veins of mice after as little as five seconds. R.V.F. virus is primarily viscerotropic in mice, and intracerebral inoculation is merely a convenient method of putting virus into the blood stream.

TABLE III.—*Brain and Blood Titres in Individual Mice Given the Same Inoculum either Intracerebrally or Intravenously.*

Route of inoculation.	Death time in hours.	Blood titre.	Brain titre.	Blood-brain titre difference.
Intracerebral	48	8·5	7·0	1·5
,,	46	>8·5	7·7	>0·8
,,	53	>8·5	6·7	>1·8
,,	86	7·8	6·3	1·5
,,	110	8·0	6·2	1·8
Intravenous	45	8·4	6·4	2·0
,,	45	>8·5	7·4	>1·1
,,	50	7·7	6·2	1·5

Titration reproducibility.—Serum from each of 5 virus-sick mice was titrated four times intracerebrally with separate sets of dilutions (6 adult mice per group, and $10^{0\cdot5}$-fold dilutions). Results in Table IV show that the maximum titre variation between mice was $10^{0\cdot7}$ and that between different titrations of a given mouse blood $10^{0\cdot5}$. Variations in titre of more than $10^{0\cdot5}$ are thus unlikely to be due to titration inaccuracies. A more accurate estimate of titration reproducibility was made when Lauffler and Miller (1944) determined the standard deviation for influenza infectivity titrations in mice, using their data, and those of Horsfall (1939). Five mice were inoculated with each tenfold dilution of virus, and it was shown that when end-points were calculated from a combination of the extent of lung consolidation and deaths, the standard deviation was $10^{0\cdot26}$. Titration

differences of $10^{0.73}$ would therefore be significant in 19 cases out of 20. Similar standard deviations ($10^{0.26}$ and $10^{0.23}$) were obtained by Knight (1944) and von Magnus (1951), when influenza virus was titrated in chick embryos, inoculating five embryos with each tenfold dilution of virus.

TABLE IV.—*The Reproducibility of R.V.F. Virus Intracerebral Titrations in Mice.*

Mouse.*	Log $_{10}$ LD_{50}/0·03 ml.			
1	8·4	8·2	8·4	8·4
2	8·8	8·7	8·6	8·7
3	8·5	8·7	8·2	8·6
4	8·2	8·7	8·5	8·5
5	8·8	8·9	8·7	8·6

* Each mouse had received an identical inoculum.

The distribution of virus in the mouse

Blood.—Pooled blood was obtained from 3 mice sick after R.V.F. virus inoculation and titrated. It contained $10^{9.3}$ LD_{50}/0·03 ml. The plasma was separated and titrated ($10^{9.7}$) and the cells washed four times in normal saline and then titrated ($10^{8.3}$). The greater part of virus in the blood is thus associated with the plasma, although the cells seem to contain more than can be accounted for by contaminating plasma. It was then shown that the age of the mouse does not affect the peak blood titre attained. Mice 2, 4, 6, 8 and 10 weeks old were given the same inoculum and when sick the blood of each mouse was titrated. Since titres were low (Table V), " incomplete "virus had no doubt been produced and it is shown in Paper IV that mice may vary considerably in peak infective titre when this happens. In spite of this, the scatter in the mean titres between age groups is small, suggesting that the final blood titre is about the same for mice between the ages of 2 and 10 weeks.

TABLE ' *eak Blood Virus Titres in Mice of Varying Age, following Identical Inocula.*

	Age of mice (weeks).				
	2.	4.	6.	8.	10.
Titres in individual mice	7·9	7·0	7·2	7·2	7·3
	7·9	7·5	7·6	7·2	7·6
	7·4	6·8		7·5	7·2
Mean titre	7·7	7·1	7·4	7·3	7·4

Organs.—In several experiments it was discovered that neither kidney, liver, brain, spleen nor lung had higher titres than the blood. Liver titres were almost as high as serum titres, and it is shown in Paper IV of this series that under exceptional circumstances they may even be higher than blood titres, but brain titres, for instance, were 10–100-fold lower. Moreover titres in the liver, kidney and spleen were even lower when, after mice had been heparinised and sacrificed, warm saline was flushed through the portal vein or aorta until the emerging fluid was clear. It would be extremely difficult to wash out all the blood from an organ, or even to remove the same amount each time. The efficiency of removal would

inevitably vary on different occasions, and very small traces of such high titre blood left behind might appreciably affect the organ titre. How, then, could one distinguish between the virus content of an organ and the virus content of the blood in the organ? It was concluded that growth curves for virus in different organs might be very difficult to construct and interpret, and, instead, a study was made of the occurrence of virus in various body secretions and excretions, and in embryos.

Urine.—In preliminary experiments urine obtained from virus-sick mice by suprapubic puncture contained virus, killing all inoculated mice in 2–5 days. There were no deaths in mice inoculated with normal urine. Subsequently, in case the needle puncture had enabled virus from blood to enter the urine, urine was expressed by abdominal pressure and, after the addition of antibiotics, inoculated into mice. There were no deaths in two of these experiments with pooled urine obtained with no-needle puncture, and in the remaining three never more than an occasional mouse died. It was concluded that there was little if any virus in the urine. This was surprising, since normal urine probably contains a few red cells and a little protein and, with such enormous blood virus titres, some spill-over into the urine seemed inevitable. A little infective mouse serum together with antibiotics was therefore added to normal mouse urine and the same amount of the same serum to Sørensen-buffered bovine albumin. After 1½ hr. incubation at 37·5° it was found that the virus in urine had a titre 10,000-fold lower than the control. Virus is thus unstable in mouse urine at body temperature. Urea is known to inactivate yellow fever (Höring, 1939), as well as other viruses (Tyrrell and Horsfall, 1954; Lauffler, Wheatley and Robinson, 1949) and in addition stability is influenced by pH. An experiment was therefore carried out, in which the same amount of an infective serum was added to the same volume of various Sørensen-buffers, and to pH 7·0 Sørensen-buffered bovine albumin, saline, and 0·3 and 3 per cent urea solutions. After incubations for 3 hr. at 37·5°, each was titrated. It seems (Table VI) that the virus is unstable when the pH is less than 7, and at pH 6 the titre falls even after 30 min. Virus, however, was equally stable for 3 hr. in Sørensen's buffer alone, or in bovine albumin, saline and even urea as long as the pH was between 7 and 8. Numerous determinations with paper indicators (B.D.H.) showed that normal mouse urine usually has a pH of from 4 to 6, and is only rarely as high as 7. Any virus escaping into such urine would therefore be inactivated fairly rapidly.

TABLE VI.—*Effect of Urea and pH on Virus Stability at* 37·5°.

					pH 7·0 buffered.			
pH 6·0 (½ hr.).	pH 6·0 (3 hr.).	pH 6·5.	pH 7·2.	pH 8·0.	0·75 per cent NaCl.	0·5 per cent bovine albumin.	0·3 per cent urea.	3·0 per cent urea.
4·3	<0*	3·8	4·8	5·0	5·4	5·4	5·4	5·5

* *i.e.*, 0·03 ml. of undiluted liquid did not kill inoculated mice.

Milk.—Two newly-delivered mothers were inoculated with virus and became sick 37 hr. later; several of their infants' stomachs were then emulsified and lightly centrifuged to obtain milk, and the supernatant inoculated with antibiotics into mice. A few of these infants were placed with new mothers but they did not die,

nor did any mice inoculated with stomach contents. Since infants had been observed to take milk shortly before or just after their mothers became sick it was concluded that little if any virus appears in the milk.

Nasal mucosa.—The nasal mucosa was tested because Francis and Magill (1935) isolated R.V.F. virus from the nasal mucosa of infected laboratory workers. Nasal washings were taken from four newly dead infected mice by introducing into the nostrils 1·0 ml. of 0·5 per cent bovine plasma albumin in saline, and collecting the fluid as it came into the mouth. The washings were in each case quite clear, and, after centrifugation, the deposit was examined for red blood corpuscles. The supernatant, after the addition of antibiotics, was titrated. Nasal washings from three of the mice tested contained an occasional lymphocyte and epithelial cell, but no red blood corpuscles ; the titres of these washings were $10^{2\cdot6}$, $10^{1\cdot8}$ and $10^{3\cdot0}$. Washings from the fourth mouse contained a few red blood corpuscles and had a titre of $10^{5\cdot0}$.

Epistaxis is common in R.V.F.-infected mice, but it appears that appreciable amounts of virus are present on the nasal mucosa when there is no microscopical evidence of haemorrhage. In the one case where a small haemorrhage had occurred titres were higher still.

Embryos.—In preliminary experiments where pregnant mice 3–4 days from term were inoculated with virus, embryos removed at the time of sickness contained virus. These embryos were not washed, and, with such high blood titres, it was thought that they might become contaminated with virus as they were removed through the uterine incisions. In subsequent experiments, two or three embryos were pooled, washed thoroughly (6 or 8 times) in normal saline before being emulsified, and mothers' blood was titrated at the time of sacrifice. Results (Table VII) show that virus is present irregularly, and in any case in low titres, in embryos. Abortions were not observed to occur. To ensure that virus was capable

TABLE VII.—*The Presence of R.V.F. Virus in the Embryos of Sick Infected Pregnant Mice.*

Pregnant mice.	Maternal blood titre.	Mice inoculated with pooled embryo suspensions.
Two at 3–4 days from term	—	SSSSSS
	—	SSSSSS
One, 16 days pregnant	—	335SSS
Four at 3–4 days from term	>8·0	123SSS
	>8·0	222333
	7·0	3SSSSS
	7·6	5SSSSS
Three at 3–4 days from term	7·4	SSSSSSSSSSS
	7·5	3SSSSSSSSSS
	6·6	33SSSSSSSSS
Two, 12 days pregnant	>8·0	22333
	7·1	335SS
Two at 3–4 days from term	8·5	Embryo titre 2·2
	7·7	„ „ >2·0

Each S or numeral indicates survival or the day of death respectively of an inoculated mouse.

of growing in embryos, laparotomies were performed under ether anaesthesia on two pregnant mice 3 or 4 days from term, and in each case two embryos given small virus inocula through the uterine wall. Twenty-nine hr. later the mothers were sacrificed and their blood as well as the inoculated embryos (washed and emulsified as before) titrated. Maternal titres were $10^{2\cdot2}$ and $10^{4\cdot8}$ $LD_{50}/0{\cdot}03$ ml., but both embryo titres were greater than $10^{5\cdot0}$. It is concluded that virus had multiplied in the embryos, and the maternal titre is accounted for by multiplication in the mother following the inevitable small leakage of virus from the embryos along inoculation tracks.

Bile.—Since normal mouse bile kills intracerebrally, even when diluted 1 in 6, the pooled bile from 6 virus-sick mice was diluted tenfold and inoculated intravenously (0·1–0·2 ml.) to 6 mice. Three died virus-deaths, and seitz-filtrates of liver suspensions killed inoculated mice in 2–3 days ; the rest survived. Thus only small amounts of virus are present in bile, such as might even have come from the blood through the puncture hole made in the gall bladder wall. To see whether bile inactivates virus, the same amount of virus was added to normal mouse bile and to the usual diluent, and incubated at 37·5° for $1\frac{1}{4}$ hr. The virus in bile was then found to have a titre one-hundredth as high as the control.

Sickness and death in mice.

Virus-sick mice adopt a hunched position, do not move much and may be hyperirritable, squeaking readily and giving small vertical hops when disturbed. They sit apart, instead of bunched together as usually. Breathing becomes more easily visible, and perhaps laboured, but stays at the usual rate of from 150–250 a minute. Later, mice frequently become hyperactive, and leap about in the box for a minute or two before the convulsive movements and the few gasping breaths which end in death. The interval between the onset of sickness and death is always very short. It never lasts more than 2 or 3 hr., and when very large inocula are given mice die after less than 30 min. sickness (see Paper III).

The relation between the virus dose and the survival time is described and analysed in Paper III of this series. Most deaths occur within 2–3 days of inoculation.

In two experiments it was shown that the response to inoculation is either death or survival with no immunity. Many mice were given 3–4 limiting dilution inoculations of stock infective sera intracerebrally at 10–20-day intervals. None of the few survivors, when bled a month after the last inoculation, had neutralising antibodies. There would therefore appear to be no intermediate responses where infected mice survive and become immune. The result of the experiment was the same, whether low or high titre serum was used for the inoculations, although low titre infective sera are shown in Paper IV to contain " incomplete " virus. In Paper IV it is also shown that a very exceptional type of inoculum may occasionally result in survival with immunity.

Rectal temperatures were determined for 34 normal mice under light ether anaesthesia, and in addition hourly determinations made on 5 mice during the course of the daylight hours. The mean value was 35·1° with a scatter of from 34–37°, and there were no particular diurnal fluctuations. Temperatures were then taken after virus inoculation, and it was noted not only that sick mice do not develop pyrexia, but that there may even be a fall to temperatures as low as

32–33°, as mice become sick. The same thermometer was used for all the temperature measurements.

Hypopyrexia in mice following the inoculation of bacterial antigens has been described by Anderson and Broderson (1948), and by Zahl and Hunter (1944). It may be that this is the usual response to antigens, but in R.V.F. infection, the fall in body temperature is at least partly a result of general hypoactivity, and the tendency of mice to sit apart from one another when sick.

Clinical pathology

There was no change in haemoglobin (Sahli) in sick mice, and pooled urine from 10 sick or newly dead mice contained some albumin (as determined by boiling), but no visible blood, no bilirubin (iodine test) and no bile salts (Hay's test). There was an amorphous sediment with a few epithelial cells but no blood cells.

Blood sugar.—If hypoglycaemia occurs in sick mice it is not a primary or only cause of sickness and death, for intravenous glucose did not noticeably affect sick mice, who died over the same time interval as did controls.

Clotting time.—While sick mice were being bled during routine virus harvesting it was noticed that blood did not clot readily, and a more detailed study of the phenomenon was therefore made. The clotting times of blood from ten normal mice were determined by taking blood from the tail into a capillary tube, and breaking off lengths of tube until a stringy clot was seen between the broken ends. The normal tail blood clotting times were all found to be 4 min. or less when this method was used. Times were then determined for 6 virus-sick mice. Values were very much greater than normal, ranging from 10 to more than 70 min., and in one case blood never clotted. It was thought possible that a large amount of virus in the blood might by its very presence interfere with blood coagulation. Therefore 2 mice were given $10^{9 \cdot 8}$ LD_{50} of virus intravenously, so that their blood titres were straight away greater than $10^{8 \cdot 0}$ LD_{50}/0·03 ml., but clotting times remained normal during 6 hr. of observation. Abnormalities in blood coagulation thus do not appear except in association with virus growth, and determinations were made on a number of mice as they became sick 50–80 hr. after the inoculation of virus. The increase in clotting time did not occur until just before or just after sickness was first detectable. There was normal clotting when normal mouse blood was mixed with three times the volume of blood from virus-sick mice. Thus the defect is caused by a shortage of something rather than by the presence of a coagulation inhibitor. Further investigations into the nature of this clotting defect are in progress.

Pathology

Important features were described by Daubney and Hudson (1931), and Findlay (1931, 1933), and a few further observations on haemorrhagic phenomena are recorded here. These phenomena were of variable occurrence and usually took place late in infection when mice were already sick. Epistaxis, which was often agonal, might occur in 4 of 6 mice in a single box, or not at all in 50 boxes. Haemorrhages into the gut occurred occasionally although they were never seen above the jejunum, and bright red blood was sometimes passed rectally. Haemoperitoneum also occurred with no obvious site of bleeding. Haemorrhagic areas in the lungs were common, and of 50 mice which had died virus-deaths, 18 had haemorrhagic

spots and blotches visible on the lung surfaces, commonly at the bases. Five mice had lungs of normal appearance, but the lungs of all the others, including those with haemorrhages, were congested with a surface appearance varying from a fine mottled pink to a deep red.

DISCUSSION

Mice dying of R.V.F. virus infection have extremely high blood titres. Levels of $10^{8 \cdot 5}$ LD_{50}/0·03 ml. (or about 10^{10} LD_{50}/ml.) are among the highest attained in tissues infected with any plant or animal virus. Nevertheless little or no virus appears in the urine, milk or bile and only to a small extent in the embryo. It is possible that there is a true excretion of virus on to the nasal mucosa, although it is difficult to be quite sure that a minute haemorrhage had not occurred, in spite of the absence of red blood corpuscles from the nasal washings.

The virtual absence of virus from urine and bile is explained by the inactivation of any virus which reaches these fluids. The action of bile is probably due to contained bile salts. Smith (1939) made a study of the susceptibility of viruses to bile salts ; it seems probable (from more recent work) that although arthropod-borne viruses are susceptible, viruses which infect by the gastro-intestinal route are relatively resistant to the action of bile.

Virus isolated from embryos may well have resulted from minute blood leakages, since haemorrhagic phenomena are common in infected mice. Haemorrhages do not occur until infection is well advanced in the mother, and blood titres are high. A small leakage of virus across the placental barrier would be adsorbed by susceptible embryo cells in the usual way (see Paper II), but before any noticeable multiplication of virus in the embryo the mother would be dead. Findlay (1931) showed that washed mouse embryos from sick mothers contained virus, in that the embryo emulsion killed all of four mice inoculated, but the matter was not pursued further. Particular attention has been paid here to embryos, because abortions in sheep and cattle were a marked feature of the original outbreak in the Rift Valley. Although aborted foetuses were not then tested for the presence of virus, virus has on occasions been isolated from the organs of aborted bovine foetuses by workers at the Kabete Veterinary Research Laboratory, Kenya, during the course of routine diagnosis, but no titrations were made (Dr. Piercy, personal communication).

It is conceivable that virus could enter and infect the embryo and thereby produce an abortion before the mother died, but it is unlikely in view of the results obtained here with mice. It seems more likely that a severe febrile illness together with haemorrhagic phenomena in a cow or ewe is enough to produce an abortion of a non-specific nature.

Blood virus titres of lethal magnitude are produced straightaway after massive intravenous injections of fresh infective blood or serum, but mice are unaffected for many hours (see also Paper III). There is thus no evidence that virus particles are themselves toxic, or that toxic substances are produced in the blood. It is tempting to attribute sickness and death to some consequence of the extensive liver damage which occurs in infected mice. Following experimental hepatectomy, hypoglycaemia is the cause of death, but if R.V.F.-sick mice are hypoglycaemic their condition is unaffected by intravenous glucose. Hypoglycaemia, if it occurs in mice, is therefore not a sole or primary cause of sickness. The comparison with a

hepatectomised animal is in any case not strictly justified, because the damaged liver remains *in situ* in infected mice. Yellow fever-sick monkeys have been shown to have low blood sugars in the terminal stages of infection (Wakeman and Morrell, 1931), but here, too, the administration of glucose intravenously did not affect their condition. Moreover, there were no clinical effects when equally low blood sugars were produced by insulin in a normal monkey.

The coagulation defect in infected mice is the probable cause of the haemorrhagic phenomena. The defect and the haemorrhages occur when mice are sick, and tissue and organ damage as a result of virus growth is maximal. The extensively damaged liver might result in a lowered prothrombin or fibrinogen concentration. This is known to occur in man following acute liver injury, and, indeed, defects in blood coagulation have been reported in men sick with yellow fever (Vellard and Vianne, 1929): the more severe the defect the commoner were the haemorrhagic phenomena. Wakeman and Morrell (1932) reported coagulation defects in yellow fever-sick monkeys, together with lowered plasma fibrinogen levels. Smithburn (1949) noticed prolonged clotting times in yellow fever-infected galagos. Further work is in progress on the relation between the haemorrhagic events and sickness and death.

It has been shown that adult mice are susceptible to extraneurally as well as to intracerebrally inoculated R.V.F. virus, and that viraemia and death rapidly ensue. Blood titres are higher than organ titres, and there is no evidence that this viscerotropic strain of virus grows in the brain to any appreciable extent, even after intracerebral inoculation. These features, together with the clotting defect, the haemorrhagic events, the histopathology in infected livers, and the general similarities between the two viruses, all suggest that the R.V.F.-infected mouse may be used as a convenient laboratory model for the yellow fever-infected man or monkey.

SUMMARY

A titration method for Rift Valley Fever virus in mice is described and its reproducibility estimated. Blood virus titres as high as 10^{10} LD_{50}/ml. occur in virus-sick mice, and the titre of the parenchyma of vascular organs is thus difficult to determine. Adult mice are as susceptible to intravenous as they are to intracerebral virus, and brain titres are lower than blood titres. The evidence indicates that this virus is viscerotropic in mice, even when inoculated intracerebrally.

Little or no virus is detectable in urine, perhaps because it is inactivated at the pH of normal mouse urine. Only small amounts of virus were detected in bile, and bile inactivates virus. Virus is virtually absent from the milk of infected mice. It is possible that there is a true excretion of virus on to the nasal mucosa. The embryos of infected pregnant mice contain irregular but small quantities of virus, which could have passed the placental barrier at the site of a small haemorrhage.

Sickness in infected mice is described. Not only is there no fever, but there may be a fall in body temperature. Deaths occur within 2–3 days, except when very small inocula are given. The interval between the onset of sickness and death is at the most 2–3 hr., and is much shorter than this when very large inocula are given. Inoculated mice either die, or survive and remain susceptible to infection.

The clotting time is greatly prolonged as mice become sick. This clotting defect is associated with virus multiplication, and is a probable cause of the haemorrhagic phenomena.

It is suggested that the R.V.F.-infected mouse can be used as a model for the yellow fever-infected man or monkey.

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RIFT VALLEY FEVER VIRUS IN MICE.
II. ADSORPTION AND MULTIPLICATION OF VIRUS

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THE preceding paper deals with the general characteristics of the infection of mice with Rift Valley Fever virus. Titration methods were evaluated, the distribution of virus in the body of infected mice determined, and clinical and pathological findings recorded. It was suggested that the mouse infected with R.V.F. virus might be used as a convenient laboratory model for the man or monkey infected with yellow fever. A quantitative study of the adsorption and growth of virus in the mouse is reported here.

MATERIALS AND METHODS

Virus and mice and routine titrations.—As described in Paper I.

Carcass titrations.—Mice were killed with ether, and heavily contaminated parts and virus inactivating fluids were removed. Skin (including paws, pinnae and snout), the gut (from lower end of oesophagus to lower end of rectum), gall bladder and bladder urine were all removed. Mice so treated were placed in pH 7·4 buffered 0·5 per cent bovine plasma albumin (500 ml. for three mice) in a sterile blendor of the Waring type. The blendor, in a refrigerator, was run slowly for 2 min. and fast for 3 min., to give a fairly homogeneous suspension which was never more than very slightly warm. Preliminary experiments had shown that virus in normal saline was markedly inactivated in the blendor, but remained stable when bovine plasma albumin was present. The suspension, after light centrifugation, was then titrated as routinely, with the addition of penicillin and streptomycin to each dilution before inoculation.

Growth curves.—Mice from whom growth curves were constructed were inoculated intracerebrally, unless the volume of inoculum was too large to be given by this route, in which case it was given intravenously. It was shown in the preceding paper that the response to inoculated virus was the same, whether it was given by the intracerebral or by the intravenous route.

Unless otherwise stated six mice were inoculated with each $10^{0.5}$-fold dilution in growth curve titrations.

RESULTS

Blood virus growth curves after various sized inocula.—Various dilutions of stock infective sera were inoculated intracerebrally, each dilution to a group of six mice. The size of the inocula ranged from $10^{0.7}$–$10^{7.3}$ LD_{50}, and the three largest were made from the same infective serum. At intervals after the inoculation the mice in each group were bled from their tails, and the pooled blood titrated. Growth curves were thus obtained (Fig. 1), and it is seen that the rate of increase in titre was proportional to the inoculum size, being greater for larger inocula. Whatever the inoculum, however, approximately the same final titre was reached. Mice began to sicken and die 1–2 hr. after peak titres were attained, and death occurred within the next 2 hr.

On several occasions growth curves were found to be similar when constructed from bleeds from the same single mouse throughout the experiment.

Two-step growth curves.—A more detailed determination was made of blood virus growth curves following large inocula. In these experiments $10^{6\cdot0}$–$10^{7\cdot5}$ LD_{50} were inoculated intracerebrally, in the form of tenfold diluted stock infective serum. The six inoculated mice were bled from the tail at closely-spaced time intervals, and growth curves again constructed from the pooled blood titres

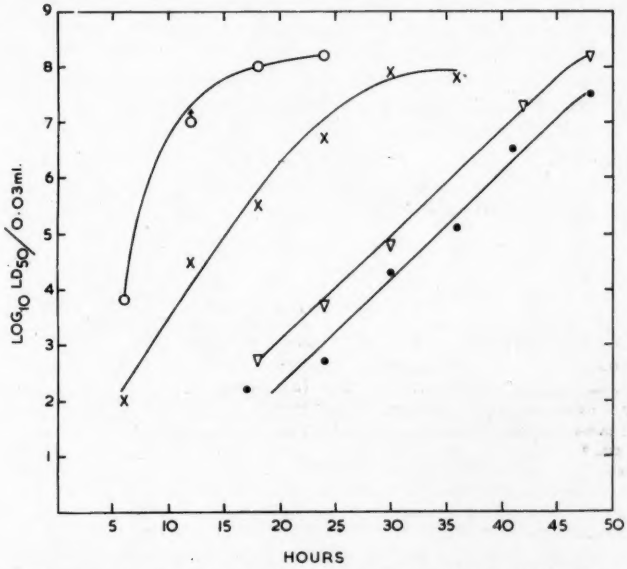

FIG. 1.—Growth curves for virus in the blood of mice following the inoculation of various doses of R.V.F. virus. Each curve was constructed from repeated samples, each one pooled, of the blood of six inoculated mice. The inocula given to each mouse were $10^{7\cdot2}$ LD_{50} (circles), $10^{4\cdot5}$ LD_{50} (crosses), $10^{1\cdot5}$ LD_{50} (triangles) and $10^{0\cdot7}$ LD_{50} (dots). Three mice only were inoculated with each $10^{0\cdot5}$ fold dilution in the titrations, except for the experiment with the smallest inoculum, where six mice were used.

(Fig. 2). In each experiment the initial rise in titre began after 7 or 8 hr., continued exponentially until the 10th hr., then remained steady or fell slightly for another hour before the second exponential rise to peak titre. Mice sickened and died after from 24 to 30 hr.

One-step growth curves.—Two more growth curves were constructed in the same way, following very large inocula. In these experiments three mice were inoculated intravenously by the tail vein, and $10^{8\cdot5}$ and $10^{8\cdot3}$ LD_{50} administered. The blood titre began to rise at 4 or 5 hr. (Fig. 3), and increased exponentially with no shelf or sag to a peak in as little as 7 or 8 hr. Mice sickened and died as the peak titre was reached, or very shortly afterwards. In one of these curves peak titres were high, and in the other peak titres were as high as occurred following intracerebral inoculation of the same material. It was repeatedly found in subsequent experiments that maximal peak titres could be produced following

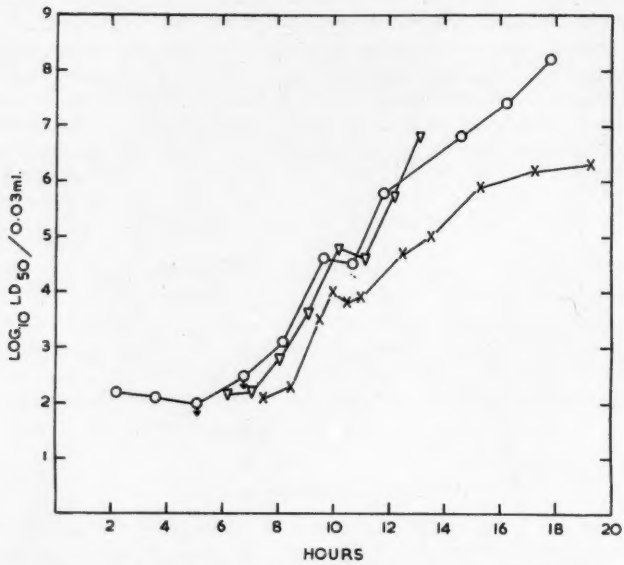

FIG. 2.—Growth curves for virus in the blood of mice following large inocula ($10^{6.0}$–$10^{7.5}$ LD_{50} per mouse). The points on each curve were obtained from repeated samples, each one pooled, of the blood of six mice which received the same inoculum.

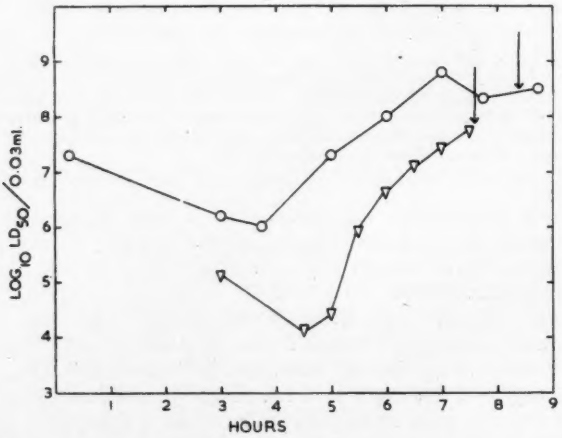

FIG. 3.—Growth curves for virus in the blood of mice following very large inocula. $10^{8.5}$ LD_{50} (circles) and $10^{8.3}$ LD_{50}/(triangles) were inoculated intravenously to three mice, and each curve was obtained from repeated samples, each one pooled, of the blood of these mice. The times of onset of sickness and death are indicated by arrows above the growth curves.

intravenous inocula. Thus, the susceptible cells which produce the virus found in blood are susceptible to intravenously inoculated virus.

Adsorption experiments.—The pattern of virus adsorption was studied in a similar way by titrating pooled tail blood from groups of mice at intervals after inoculations of various sizes. Very similar curves were obtained on many occasions, and the results of three of these experiments are given in Fig. 4.

The blood titre falls rapidly for the first hour, then less rapidly, although the fall seems to continue during the period of the experiments. The curves in Fig. 4 are drawn by eye through the determined points, and in each case, the titre has fallen by $10^{0 \cdot 6}$ within about 30 min. This means, on a log scale, that 75 per cent of the inoculum has disappeared from the blood within 30 min.

In two of these experiments inocula were made from infective sera of low titre ($10^{7 \cdot 0}$–$10^{8 \cdot 0}$). It is shown in Paper IV of this series, that such sera contain

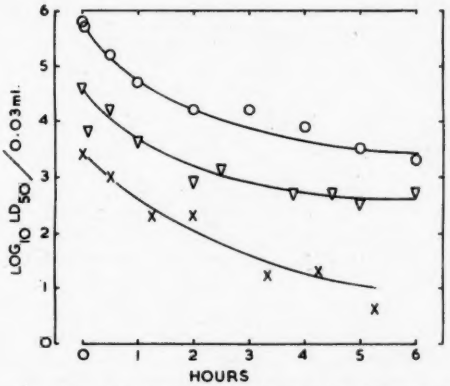

FIG. 4.—Adsorption curves for virus from the blood of mice following various sized inocula. Each mouse given $10^{7 \cdot 6}$ LD_{50} (circles), $10^{6 \cdot 4}$ LD_{50} (triangles) and $10^{5 \cdot 2}$ LD_{50} (crosses), and the theoretical immediate blood titre (calculated from the blood volume) is shown in each case. Points on each curve were obtained from repeated samples, each one pooled, of the blood of 4–6 inoculated mice.

" incomplete " virus, and the third adsorption experiment was performed with a high titre serum ($10^{9 \cdot 0}$) which probably contained little or no " incomplete " virus. In the curve obtained (triangles in Fig. 4) there is a suggestion of a steady plateau after the 4th hr., but the initial part of the curve is similar to that in the other experiments. The shapes of these growth curves, therefore, do not reflect any appreciable difference between " incomplete " and " complete " virus.

It was thought that perhaps it might, for purely circulatory reasons, take a long time for all virus particles to reach susceptible cells. If, however, virus encountered susceptible cells earlier, adsorption would perhaps be accelerated. About $10^{7 \cdot 0}$ LD_{50} of virus contained in 0·35 ml. of diluent was therefore injected very slowly (over the course of 4 min.) into the portal vein of an anaesthetised mouse, and the same dose into the tail vein of another mouse. Heart bloods were titrated after 10 min., and the titres were found to be the same ($10^{5 \cdot 9}$ and $10^{6 \cdot 1}$) in each. Thus, if liver cells adsorb virus from circulating blood, the rate

of adsorption is not markedly increased when inoculated virus enters the hepatic before the systemic circulation.

Virus stability at 37·5°.—Any apparent steadiness of the final titre in growth curve experiments could possibly represent an increase in blood titre masked by thermal inactivation of virus at the body temperature of mice. The progressive fall in titre which occurred after the second or third hour in adsorption experiments might also be due to thermal inactivation. Stock infective serum was therefore incubated at 37·5° and samples removed and titrated at intervals. The titre remained steady for many hours (Table), showing that thermal inactivation is not an important factor in interpreting the growth curves reported here. This stability at 37·5° was later confirmed, and it was shown that there was a 10-fold fall in titre by 24 hr., a 10,000-fold fall by 48 hr., and that no infective virus was demonstrable after five days. Antibiotics were added to the serum before incubation, and bacteriological cultures made at the time of each titration were sterile.

TABLE—*Virus Stability in Mouse Serum at* 37·5°

	Hours.					
	0.	1.	2.	3½.	4.	5.
Titre . .	6·3 .	6·0 .	6·4 .	6·4 .	6·3 .	6·0

Adsorption and growth curves obtained from carcass titrations.—It was thought possible that the observed changes in blood titre in the adsorption and growth curves described above might partly be due to blood-tissue exchanges of virus in the body of the mouse. Growth curves were therefore constructed from the titration of entire carcasses.

A preliminary experiment was performed to see whether virus was inactivated in suspension of normal mouse carcass. After standing at room temperature for 15 min., the titres for virus in a normal carcass suspension and the same amount in standard diluent were found to be $10^{3·9}$ and $10^{3·5}$ respectively. Normal carcass emulsion thus does not inactivate virus.

Two experiments were then done, in which adsorption and growth curves were obtained from both blood and carcass titrations. Many mice were inoculated intracerebrally with $10^{7·3}$ and $10^{7·5}$ LD_{50} of virus, and at fixed time intervals three mice were sacrificed and their pooled blood and pooled carcasses titrated. These titrations were made using serial tenfold dilutions of infective material. In the curves obtained (Fig. 5), the average virus content per mouse carcass as directly determined is plotted, together with the average virus content per mouse blood volume (Kaliss and Pressman, 1950) calculated from the blood titration. The curves are, if anything, more irregular than when successive blood samples are taken from the same set of mice throughout the experiment, but it can be seen that carcass and blood titrations give similar curves. It is concluded that adsorption and growth curves for blood virus give a true picture of the behaviour of virus in the entire mouse. In both experiments, the carcass titre begins to increase at about the same time as the blood titre, showing that this increase is not detectable anywhere in the body very long before it is detectable in the blood.

It would appear from the growth curve that the residual virus content of the carcass after adsorption is greater than that of the blood. This, however, is not the case in the adsorption curve, and it could not be confirmed in subsequent

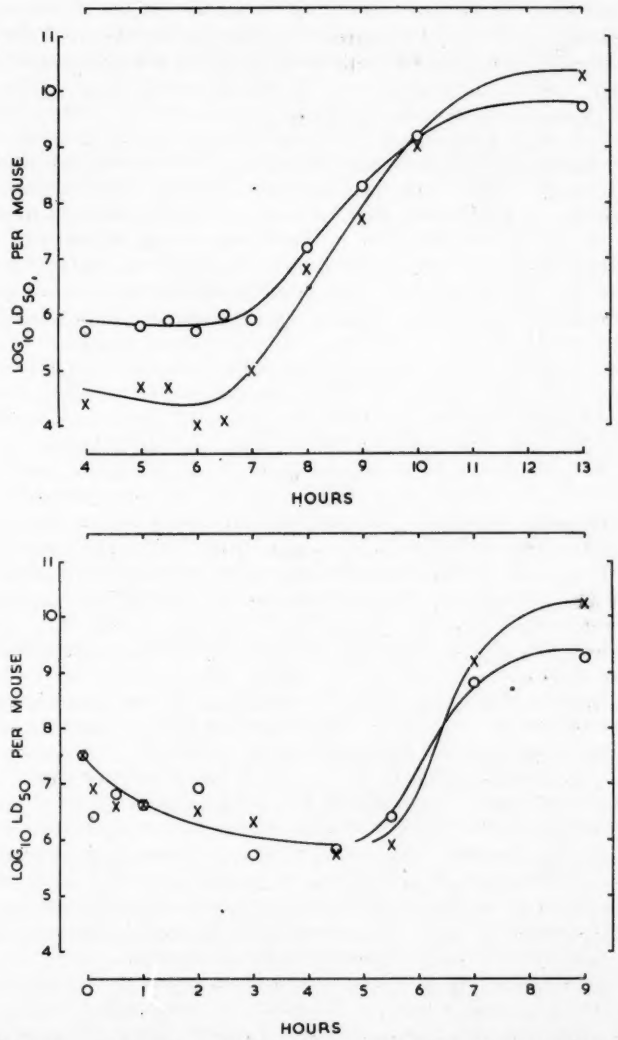

FIG. 5.—Adsorption and growth curves for virus in the carcasses of mice (circles) and in their blood (crosses). At each time interval three inoculated mice were sacrificed and their pooled blood and pooled carcasses titrated. The total virus content per mouse was determined directly in the carcass titrations, and the content per mouse blood volume was calculated from the blood titrations. $10^{7.3}$ and $10^{7.5}$ LD_{50} were inoculated per mouse, and in the adsorption experiment which terminated at 9 hr., the theoretical immediate virus content per mouse ($10^{7.5}$ LD_{50}) is shown.

experiments. The blood and tissue points on the adsorption curve are not regular enough to draw conclusions about the virus content of blood and tissues during adsorption. It can be seen in both the growth curve and the adsorption curve, however, that the whole blood usually contains about as much virus as the carcass.

DISCUSSION

These adsorption and growth curves may be interpreted as follows. After inoculation into the blood stream virus is adsorbed by susceptible cells, and the blood titre falls. It was shown in Paper I that an intracerebral inoculation is, in effect, a small intravenous one. Almost all of this adsorption takes place during the first hour, and any subsequent adsorption is, by comparison, very limited in extent. It is shown in the carcass experiments (and in Paper III of this series), that virus which has disappeared from the blood is no longer detectable even when the entire carcass is titrated. Within from 5 to 7 hr., virus which has undergone one multiplication cycle in cells is being released into the blood, raising the titre above the residual level. This increase in titre occurs exponentially, and in the experiments in Fig. 2, where the inoculum did not infect all available cells in the first place, the newly released virus is again taken up by uninfected cells. At 10–11 hr., this rate of uptake equals or even slightly exceeds the rate of release, so that the reproducible shelf or sag in the curve appears. When all susceptible cells have taken up virus, released virus can accumulate in the blood to give the final exponential rise in titre. With very large inocula (Fig. 3), which were themselves large enough to infect all susceptible cells, virus released after the first multiplication cycle can increase uninterruptedly in the blood, and the peak titre is reached after the first cycle of growth, by 10 hr. The size of a saturating inoculum, itself just large enough to infect all susceptible cells, is determined in Paper III.

Are such interpretations justified in analysing these curves? In the first place, the phenomenon, with virus disappearance and subsequent increase, has been shown to occur for other viruses on much the same time scale.* Secondly, the shapes of the growth curves in Fig. 2 and 3, which are most simply explained in these terms, are also reproducible. Thirdly, the virus disappearance and subsequent increase is detectable when the entire carcass is titrated (Fig. 5), and is thus not to be explained by any purely blood-tissue exchanges of virus. An attempt to reproduce the shelf in the growth curve (Fig. 2) with carcass titrations has not been made, because they are probably less accurate than blood titrations, and contributions to successive points on the growth curve are made by different mice, so that mouse variation becomes important.

Henle (1949) showed that when influenza virus grows in the chick embryo an increase in infectivity is detectable in the allantoic membrane a few hours before it is reflected in the allantoic fluid. For R.V.F. virus, since the growth curve for the mouse carcasses is similar to that for mouse blood, one can assume that almost as soon as virus increases in the tissues the increase is detectable in the blood, *i.e.*, virus is liberated into the blood stream soon after it is produced.

Although growth curves have been constructed for a number of viruses, discrete cycles of multiplication have been demonstrated only for a few, including bacterio-

* Stepwise increases in titre have been described for poliomyelitis virus in tissue culture, however, and in this *in vitro* system each cycle appears to last for ten days (Scherer and Syverton, 1952).

phage (Delbruck and Luria, 1942 ; Ellis and Delbruck, 1939), influenza virus (Henle, Henle and Rosenberg, 1947 ; Henle and Rosenberg, 1949 ; Cairns, 1952), pneumonia virus of mice (Ginsberg and Horsfall, 1951), Newcastle disease virus (Gordon, Birkeland and Dodd, 1952), Western Equine Encephalitis virus (Dulbecco and Vogt, 1954), and Theiler's GDVII virus (Sanders, 1953). Usually some special method has been needed to demonstrate these multiplication cycles. Where it is technically possible the medium surrounding infected cells can be removed and replaced, so that the total virus release is measured during fixed time intervals with no residual titre to confuse the picture. Methods have also been developed for preventing the re-uptake of virus after its release from the first cycle of growth in cells.

It seems that cycles of multiplication are visible in the straightforward R.V.F. virus growth curves obtained here. They are thus demonstrable in the intact virus-infected mammal. Ginsberg and Horsfall (1951) and Gordon *et al.* (1952) constructed such curves for pneumonia virus of mice in mice and for Newcastle disease virus in eggs. Von Magnus (1951) published growth curves for influenza virus in the allantoic cavity, in which a shelf or sag occurs repeatedly at 10 hr., although it is not commented on in the text. The very rapid growth rate of R.V.F. virus, together with the fact that blood from the same individuals can be titrated during the course of an experiment, may partly explain why the cyclic pattern was so clearly seen here.

Adsorption takes place fairly rapidly, and 75 per cent of inoculated virus had disappeared within about 30 min. Similar rates of adsorption have been reported for other viruses. Henle *et al.* (1947) showed that about 70 per cent of inoculated infective influenza virus was no longer detectable in the allantoic fluid after from 30 to 60 min. Dulbecco and Vogt (1954), in a precise *in vitro* experiment, showed that 80–90 per cent of Western Equine Encephalitis virus is adsorbed by a monolayer of susceptible cells within about 30 min. Gordon *et al.* (1952) reported that 87 per cent of inoculated Newcastle disease virus had disappeared from the allantoic fluid in as short a time as 10 min.

The titre remaining in the blood after adsorption is completed is related to the size of the inoculum (Fig. 1, 2 and 3). There is more virus left over when more is administered. A study of this phenomenon is made in the next paper of this series.

It can be seen from the growth curves in Fig. 2 and 3, that there is an increase in titre much earlier with large inocula than with smaller ones. The increase may occur after as short a time as 4 to 5 hr., or as long as 7 to 8 hr., after inoculation. A similar effect has been reported for other viruses. Liu and Henle (1951) showed that when the LEE strain of influenza B virus grows in the allantoic cavity, and growth curves are constructed from allantoic fluid samples, the " constant period " between inoculation and increase in titre could be reduced from 8–10 to 4 hr. by giving larger inocula. One conceivable explanation of the results obtained with R.V.F. virus is that because the rate of increase in titre is greater with larger inocula (Fig. 1), the curve will begin to rise above the admittedly higher residual titre at an earlier stage. Alternatively it is possible that with high multiplicities of infection (where many particles infect each cell) virus begins to be released from cells earlier, as has been shown for Western Equine Encephalitis virus *in vitro* (Dulbecco and Vogt, 1954). The " average liberation time ", or time between inoculation and the appearance of 50 per cent of the first cycle

yield (Fazekas de St. Groth and Cairns, 1952), is also shorter when large inocula are given. The average liberation time as judged from the one-step growth curves (Fig. 3) is about $6\frac{1}{2}$ hr., and considerably shorter than the $9\frac{3}{4}$ hr. in the two-step growth curves (Fig. 2). This is not easy to explain except by again suggesting that the intracellular latent period is shorter when a very large inoculum is given (Dulbecco and Vogt, 1954).

When large inocula were given mice sickened and died as peak blood titres were reached, or very shortly afterwards (Fig. 3). With smaller inocula sickness and death occurred 1–4 hr. after peak titres were attained. Thus, peak blood titres occur before mice become sick (except with extremely large inocula ; see Paper III), and death occurs before thermal inactivation could affect these titres.

The experiments in Fig. 1 showed that the peak virus titre was approximately the same whatever the size of the inoculum. The peak titres vary to a greater extent in the growth curves in Fig. 2 and 3, and an analysis of these variations is made in Paper IV of this series. It will be shown that although peak titres are uniformly high following any inoculum consisting of " complete " virus, titres are more irregular when " incomplete " virus is present.

SUMMARY

Adsorption and growth curves are constructed for blood titres in mice following the inoculation of various doses of Rift Valley Fever virus. After inoculation the amount of virus in the blood decreases, almost all this decrease occurring in the first hour. The virus titre then decreases very slightly until a rise is detected from 5 to 9 hr. after inoculation, depending on the amount of virus administered. The length of the average liberation period, between inoculation and the appearance of half the first cycle yield, is shorter with large inocula. The rate of increase in titre is more rapid with large inocula.

Adsorption and growth curves constructed from carcass titrations confirm the general picture of virus adsorption and multiplication, and show in addition that virus is liberated into the blood almost as soon as it is produced in the mouse.

When large inocula are given there is a shelf or sag in the growth curve at 10 hr., which recurs in separate experiments. With very large inocula, however, the rise in the growth curve is uninterrupted, reaching a peak by 10 hr. These features are explained as due to cycles of multiplication in susceptible cells of the mouse.

As long as " incomplete " virus is not present peak blood titres are much the same, whatever the inoculum size, and whether intracerebral or intravenous inoculations are given. Mice sicken and die during the first few hours after these peak titres are reached, except when extremely large inocula are given.

Virus in mouse serum is stable for many hours at 37·5°.

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RIFT VALLEY FEVER VIRUS IN MICE. III. FURTHER QUANTITATIVE FEATURES OF THE INFECTIVE PROCESS

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EARLY in the studies reported in this series of papers it was noticed that the time at which infected mice died was related to the amount of R.V.F. virus inoculated. The growth curve experiments reported in the previous paper showed that the residual virus titre after adsorption was also related to the amount of virus inoculated. A more detailed study of both these relationships is reported here, together with a determination of the size of a saturating inoculum.

MATERIALS AND METHODS

Virus and mice and routine titrations.—As described in Paper I.
Carcass titrations.—As described in Paper II.

RESULTS

The relation between dose and incubation period

A serum, of titre $10^{9 \cdot 0}$, was obtained in the course of a 10^{-4} passage series, in which harvested infective serum was diluted 10^{-4} at each passage, before inoculation into the next group of mice. In such a passage series titres are, as a rule, uniformly high, although "incomplete" virus may be present (see Paper IV). This serum was serially diluted in $10^{0 \cdot 5}$-fold steps, and each dilution inoculated into 15 mice. Each of the two largest inocula was given intravenously to a single mouse. The time after inoculation at which each mouse died was recorded, and it is seen (Fig. 1) that the median death time is inversely proportional to the logarithm of the dose inoculated. However small the inoculum, mice very rarely died more than 100 hr. afterwards. With small inocula there were often one or two mice which died 20–50 hr. after the others, but if these were not counted the deaths with any given inoculum occurred over a period of 10–15 hr. It is possible that the scatter is not more than 1 or 2 hr. when more than $10^{9 \cdot 0}$ LD_{50} are inoculated, but it has not been feasible to give such large inocula to more than a small number of mice. It is because the mean death-time is unduly lengthened by the one or two late deaths that the median value is used. This gives a better indication of the period of time over which nearly all deaths occurred.

Blood or inoculated material often leaks on to the surface of the head after intracerebral inoculation, but the scatter in death-time was of the same order when mice were inoculated intravenously. The differences in death-times after a given inoculum are thus not due to differences in the exact amount of virus deposited in each mouse. It was found that mice from the same litter were more likely to die within a short time of each other than were mice from different litters.

It seems, therefore, that the scatter in individual death-times is due to mouse variation. It is shown in Paper IV that the scatter following doses of " complete " virus is less than the scatter with inocula containing " incomplete " virus. In addition the median death time with a given infective inoculum is shown to be much later when " incomplete " virus is present.

In infectivity titrations an occasional mouse dies as long as 8–10 days after inoculation. Such deaths were shown to be due to R.V.F. virus, in that Seitz-filtered liver suspensions killed inoculated mice in 2–3 days. These late deaths, also noticed by earlier workers (Findlay, 1931), occurred in boxes where one or more mice had already died virus deaths within the usual 5 days of inoculation.

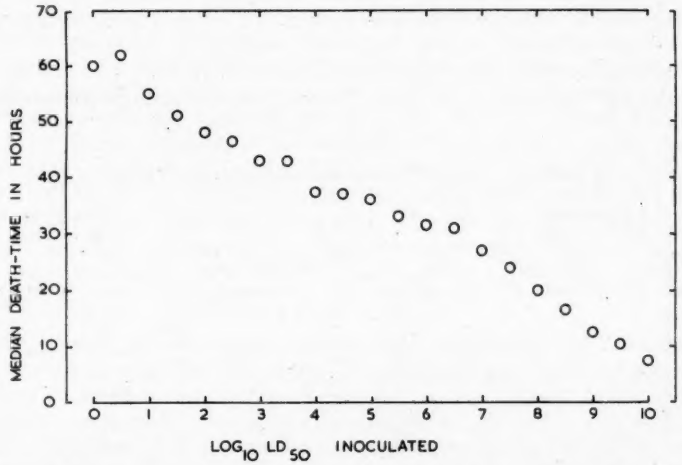

FIG. 1.—Relation between dose and death-time. Each point is the median death-time of a number of mice which were given the same infective inoculum. Fifteen mice were inoculated intracerebrally in each case, except for the two largest doses, which were given intravenously to single mice. The same infective serum was used for every inoculation in the experiment. Early traumatic deaths were ignored, and since in addition not all mice given the smallest inocula died, some of the median values were calculated from fewer than fifteen death-times.

Dead mice are sometimes partly eaten by their surviving companions before being removed at the next mouse check, and an experiment was performed to see if the cannibals could be infected orally. In each of six boxes of 6 mice one mouse only was inoculated with virus and the others starved. On the second day the inoculated mice died and 4 of them were partly or completely eaten by the survivors, which were then fed normally and observed for 5 days. In each box where an infected mouse had been eaten there were one or two virus deaths. In another experiment mice were infected when there were fairly large amounts of virus in their drinking water. The late deaths occur in boxes of mice which have received limiting dilutions of virus which would not normally kill all mice ; the phenomena could not, of course, occur when a uniformly lethal inoculum is given. In several separate experiments limiting dilutions of both high titre and low titre sera (containing " incomplete " virus, see Paper IV) were given to a total of 500 mice,

which were kept in separate boxes. They were observed for up to 20 days, but there were no late virus-deaths, and 48 mice survived. Intranasal infection from dust and débris, if it occurs, does not occur over any distance, for the boxes were adjacent. Late deaths are therefore a result of cannibalism.

The size of a saturating inoculum

A saturating inoculum itself infects all susceptible cells so that the growth curve is of the one-step type (see Paper II), and mice sicken and die as the peak titre is reached or shortly afterwards. If peak titres are attained by 10–11 hr. and deaths occur on the average within, say, 16 hr. of inoculation, one can probably assume that the growth curve was of the one-step kind, and that the inoculum was a saturating one. In order to determine the size of a saturating inoculum mice were given very large inocula of various sizes and death-times recorded.

The results of several experiments are included in Table I. In another experiment where 10-hour blood titres were also determined, each inoculum was given

TABLE I.—*Death Times Following Large Inocula.*

Titre of serum used (log_{10} LD_{50}/0·03 ml.).		Log_{10} LD_{50} given.	Number mice inoculated.	Death time in hours.*	
High titre sera.	Low titre sera.			High titre sera.	Low titre sera.
9·0	—	10·0	1	7·5	—
9·0	—	9·5	1	10	—
8·3	—	9·3	3	7	—
8·1	—	9·0	1	6	—
9·0	—	9·0	15	12·5	—
8·4	—	8·9	3	9	—
8·3	—	8·8	5	8	—
9·0	—	8·5	13	16·5	—
—	7·4	8·4	15	—	10·6
8·3	—	8·3	5	13	—
—	7·2	8·2	3	—	14
8·1	—	8·1	8	13	—
9·0	—	8·0	4	17	—
9·0	—	8·0	13	20	—
8·9	—	7·9	6	17	—
8·3	—	7·8	3	17	—
—	7·2	7·7	3	—	17
—	6·6	7·6	3	—	14
8·7	—	7·7	6	19	—
9·0	—	7·5	16	24	—
8·5	—	7·5	3	17·5	—
—	7·4	7·4	18	—	21
8·3	—	7·3	3	19·5	—
—	7·2	7·2	3	—	21
8·1	—	7·1	9	18	—
8·1	—	7·1	2	24	—
9·0	—	7·0	13	27	—
8·0	—	7·0	7	23	—
—	7·4	6·9	18	—	26
8·3	—	6·8	3	23	—
—	7·2	6·7	3	—	25
9·0	—	6·5	18	31·5	—
—	7·4	6·4	18	—	32·7

* The median death time is given except when four or less mice were inoculated, when the mean is used.

TABLE II.—*Death Times and Ten-hour Blood Titres following Large Inocula.*

Log_{10} LD_{50} given.	Mean death time in hours.	Log_{10} LD_{50} per 0·03 ml. blood at 10 hr.
9·6	10·4	9·6
9·1	12·3	9·8
8·6	14·2	9·5
8·1	16·5	8·5
7·6	19·7	7·4
7·1	22·5	6·4
6·6	25·0	5·9

to 3 mice, whose pooled tail blood was titrated 10 hr. later (Table II). In this experiment the same infective serum was used for all the inoculations, and the peak titre of $10^{9·5}$–$10^{9·8}$ was attained within 10 hr. after the three largest inocula. It can be seen from both types of experiment that inocula containing more than $10^{8·0}$–$10^{8·5}LD_{50}$ produce peak titres within 10 hr., and an average death time of less than 16 hr. Inocula of this size are therefore saturating ones. In the experiments where death times were recorded, inoculations which were made from sera of titre less than $10^{8·0}$ are listed separately, because it was shown later that such sera contain " incomplete " virus (see Paper IV). The results, nevertheless, appear to be the same whether low titre or high titre sera were used.

Relation between dose and adsorption

A stock infective serum was titrated (9 mice inoculated with each serial $10^{0·5}$-fold dilution) and each of the $10^{0·5}$-fold dilutions then inoculated into 3 randomised mice. The two largest inoculations were given intravenously so that maximum doses could be included. Pooled blood from each group of 3 mice was titrated after 5 hr., by which time adsorption was considered to be completed (see Paper II). Since the titre may begin to increase earlier than this with large inocula, these were titrated in addition after 4 hr. Virus present in the whole mouse blood was calculated by multiplying the amount of virus per 0·03 ml. of blood (the titration figure) by 68, assuming that the blood volume of the 16 g. mice used was 2·0 ml. (Kaliss and Pressman, 1950). Subsequently, whole mouse carcasses were titrated as described in Paper II, following various inocula, and in two final experiments bloods and whole mice were titrated following extremely large inoculations of high titre serum. It was found that a rise in titre took place between the fourth and the fifth hour with some of these inocula, and even earlier when some of the extremely large inocula were given. The lowest figure was in each case used to calculate the virus content per mouse after adsorption. The results from all these experiments are included in Table III, and represented graphically in Fig. 2. The points relating amounts of virus inoculated to amounts left over after adsorption appear to form a straight line. The coefficient of correlation, r, was computed from all data, and found to be $0·968 \pm 0·208$. The value of t at $P = 0·001$ is only 3·792 for 22 degrees of freedom, but t calculated from the data obtained was found to be 18·09. The degree of correlation was thus extraordinarily close, and the result is highly significant.

The regression equation was calculated from the data in Table III ($x = 0·74y + 2·97$) and the regression line is drawn through the points in Fig. 2. The relation

between the amount of virus inoculated and the amount adsorbed was calculated from the regression equation, and is shown graphically in Fig. 3. The amount adsorbed is expressed both in $\log_{10}LD_{50}$, and as a percentage of the amount inoculated. It can be seen that when $10^{9\cdot0}$ LD_{50} are administered more than 85 per cent of this is adsorbed, but with larger inocula the percentage falls markedly. The largest inoculum given experimentally was $10^{10\cdot5}$ LD_{50}, and the calculated

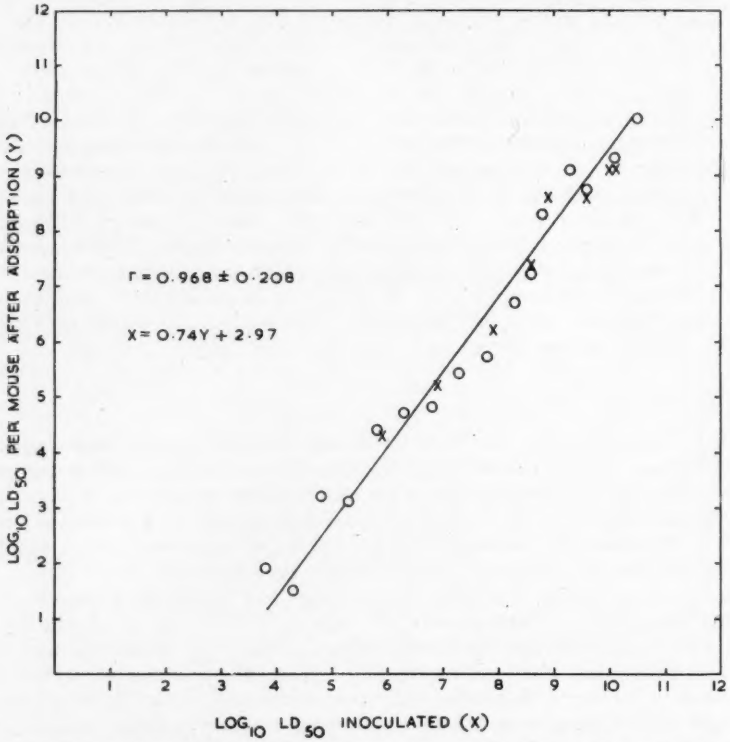

FIG. 2.—Unadsorbed residue (y) following various sized Rift Valley Fever virus inocula (x). Data from Table III, including figures obtained from blood titrations (circles), and carcass titrations (crosses). Coefficient of correlation (r) = 0·968 ± 0·208. Regression equation : $x = 0\cdot74y + 2\cdot97$. The regression line is drawn through the points.

amount then adsorbed is $10^{10\cdot2}$ LD_{50}. If the adsorption for inocula larger than this is calculated, maximal amounts of $10^{10\cdot5}$ LD_{50} are adsorbed. The regression equation cannot in any case be used for inocula greater than about $10^{11\cdot1}$ LD_{50}, because the calculated residue then becomes larger than the inoculum itself.

A further conclusion can be drawn from the results of these adsorption experiments. Whether the quantity of virus left in a mouse after adsorption is calculated for blood from the blood titration or determined directly by carcass titration the figures obtained fall on the same straight line. After adsorption, therefore, there is

TABLE III.—*Adsorption following Various Sized R.V.F. Virus Inocula.*

Inoculum ($\log_{10}$ LD_{50}) (x).	Number of mice.	Sample.	Sample titre.	Calculated $\log_{10}$ LD_{50} virus per whole mouse (y).
10·5	1	3 hr. blood	8·2	10·0
10·1	1	3 hr. carcass	5·2	9·0
10·1	1	2 hr. blood	7·5	—
		3 hr. „	7·5	9·3
10·0	3	3 hr. carcass	5·4	9·1
9·6	2	3 hr. blood	6·9	8·7
9·6	2	3 hr. carcass	4·8	8·6
9·3	3	4 hr. blood	7·3	9·1
	3	5 hr. „	8·1	—
8·9	3	4 hr. carcass	5·1	8·6
8·8	3	4 hr. blood	6·5	8·3
8·6	2	3 hr. „	5·4	7·2
8·6	2	3 hr. carcass	3·6	7·4
8·3	3	4 hr. blood	5·2	—
	3	5 hr. „	4·9	6·7
7·9	3	5 hr. carcass	2·5	6·2
7·8	3	5 hr. blood	4·9	6·7
7·3	3	5 hr. „	4·6	6·4
6·9	3	5 hr. carcass	1·5	5·2
6·8	3	5 hr. blood	3·0	4·8
6·3	3	5 hr. „	2·9	4·7
5·9	3	5 hr. carcass	0·6	4·3
5·8	3	5 hr. blood	2·6	4·4
5·3	3	5 hr. „	1·3	3·1
4·8	3	5 hr. „	1·4	3·2
4·3	3	5 hr. „	—†	1·5
3·8	3	5 hr. „	0·1	1·9

In each experiment the infective serum used was of a titre greater than $10^{8\cdot0}$.—The largest inocula were given intravenously.

Coefficient of correlation (r) = $0\cdot968 \pm 0\cdot208$.

Regression equation : $x = 0\cdot74y + 2\cdot97$.

† One of the six mice inoculated with undiluted blood died a virus death.

as much virus in the blood as there is in the entire carcass, and it is concluded that at this time virtually all infective virus is contained in the blood.

It can be seen in Fig. 2 that when nearly all the inoculated virus is adsorbed the small amount remaining is proportional to the inoculum size. An experiment was performed to see whether this unadsorbed residue was normally adsorbed in another mouse. Three mice were given $10^{7\cdot4}LD_{50}$ of a high titre serum intravenously, and 5 hr. later the mice were bled out, and their pooled serum titrated and immediately inoculated (0·1 ml. intravenously) into each of 3 more mice. These 3 mice in turn were bled, and their pooled serum titrated at 5 hr. It was found that the virus left over after adsorption was adsorbed by the second group of mice to the extent which would be expected from the dose-adsorption data already obtained. This experiment was repeated with the same result.

The effect of a supersaturating inoculum

R.V.F. virus, when very large doses are inoculated, will kill mice in as little as 7–8 hr. (Paper II, Fig. 3), and it was shown that by this time virus which has multiplied in cells is already being released into the blood. These extremely large inocula are themselves able to infect all susceptible cells, and if damage to cells as

a result of virus growth is a cause of death mice presumably should not die until a single cycle of growth is nearly completed. Thus, unless enormous concentrations of virus particles are themselves toxic, death should not occur until at the earliest 4–5 hr., however large the inoculum. A mouse was therefore given repeated large intravenous injections of undiluted high titre sera ($10^{8\cdot5}$–$10^{9\cdot5}$ LD_{50}/0·03 ml.). A total of about $10^{10\cdot7}$ LD_{50} were given, contained in 1·6 ml. serum, over the course of $1\frac{3}{4}$ hr. At 2 hr., the blood titre was $10^{8\cdot3}$ LD_{50}/0·03 ml. but nevertheless the mouse remained perfectly well until 6 hr., when it died with almost no preceding sickness.

DISCUSSION

The relation between the logarithm of the amount of virus administered and the logarithm of the amount of virus left over after adsorption appears as a straight line. It was shown that after adsorption the blood of a mouse has the same total virus content as does the carcass. This means that virtually all virus present in the mouse is then contained in the blood, and that virus adsorbed from the blood is no longer detectable. Even when nearly all the inoculated virus disappears, however, the size of the small unadsorbed residue is proportional to the size of the inoculum. It is conceivable that a small percentage of infective particles differ from the rest in that they are poorly adsorbed. There would be more of these particles present in larger inocula, so that larger amounts of virus would be left unadsorbed after 5 hr. This residue, however, was found to be adsorbed to the normal extent when inoculated without delay into other mice. Unadsorbed virus therefore does not differ in this respect from normal virus.

The calculations from the regression equation derived from the data in Table III showed that a mouse can adsorb at least $10^{10\cdot2}LD_{50}$ of virus (Fig. 3). It was shown, however, in other experiments reported in this paper, that an inoculum of $10^{8\cdot0}$–$10^{8\cdot5}$ LD_{50} itself just infects all available cells. If the disappearance of such large extra amounts of virus is not to be explained by some physical phenomenon like the clumping of virus particles, it means that susceptible cells can adsorb 50–150 times the quantity of virus contained in a saturating inoculum. Possibly a given cell is able to adsorb 50–150 times the number of particles needed to infect it.

Henle (1949) showed that when influenza virus was inoculated into the allantoic cavity 70 per cent of the inoculum was no longer detectable in the allantoic fluid after adsorption. The percentage which disappeared was approximately the same, whether $10^{2\cdot0}$ or $10^{9\cdot0}EID_{50}$ were inoculated. Inocula as large as $10^{9\cdot6}$ were given but there was no evidence that a smaller percentage was adsorbed than with smaller inocula.

The median death time in inoculated mice has been shown to be inversely proportional to the logarithm of the infective dose. A similar straight-line relationship between incubation period and the logarithm of the dose inoculated has been shown to occur for influenza virus administered intranasally to mice (Horsfall, 1939), and for papilloma virus administered subcutaneously to rabbits (Bryan and Beard, 1939). Presumably, since the incubation period is determined by a process (viral multiplication) which occurs at an exponential rate, it is dependent on an exponent of the amount of virus which initiates the multiplication process; that is to say, on an exponent of the size of the inoculum. Nearly all deaths with any

given inoculum occur during a period of 10–15 hr. It seems probable that this variation in death time following a given virus dose is due to individual differences in mice. There may be slight variations in virus growth-rate in individual mice, or alternatively mice may vary in the time they take to die with a given growth-rate.

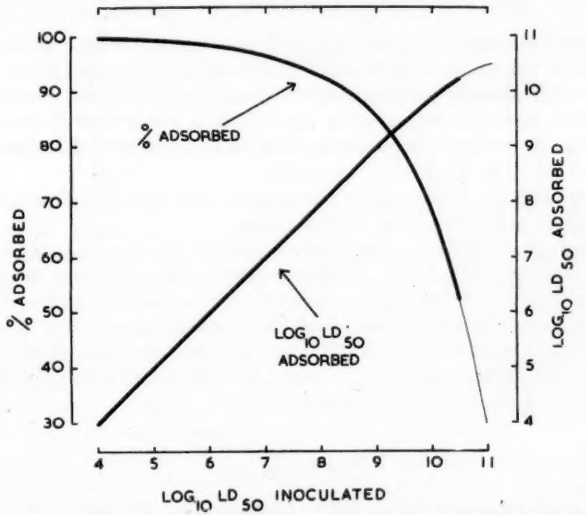

FIG. 3.—Relation between amount of virus inoculated and amount adsorbed, calculated from the regression equation ($x = 0{\cdot}74y + 2{\cdot}97$) which was obtained from the data in Table III. Those parts of the curves calculated for inocula larger than were given experimentally are represented as thin lines.

It was shown in Paper II that the cycle time as expressed by the " average liberation time " (Fazekas de St. Groth and Cairns, 1952) is shorter when very large inocula are given. The shortest " average liberation time " obtained in the growth curve experiments reported in Paper II was 6½ hr. The mouse given the supersaturating inoculum described above, therefore, died after the minimum single cycle time, and although the total virus yield may not by then have been released from cells, cell damage would probably be maximal.

Quantitative details of virus behaviour such as extent of adsorption, length of a single cycle of growth, or size of a saturating inoculum, have in the past only been determined for viruses like influenza and bacteriophage. Workers with these viruses have a well-defined virus-host system, which comprises a known number of susceptible cells of a uniform type surrounded by a fluid of known composition which can be altered or replaced as required. For R.V.F. virus in mice, however, the susceptible cells are unknown in number, type or site, and the extracellular fluids have a complex and uncontrollable composition and motion. All the unknown and no doubt intricate reactions of the host to a virus infection contribute to the picture. Nevertheless many of the fundamental properties of viruses growing under simpler and more precise conditions, are apparently paralleled in the virus-infected mammal. Although in these studies

details of the cell-virus interaction cannot be analysed so precisely, it is of importance to know that the phenomena described in the allantoic cavity and *in vitro* may also occur in the complex intact host.

SUMMARY

The relation between inoculum size and the extent of adsorption is described. The logarithm of the amount of unadsorbed virus is proportional to the logarithm of the amount inoculated. It is calculated that a single mouse can adsorb at least $10^{10 \cdot 2}LD_{50}$. The size of a saturating inoculum is determined, and found to be $10^{8 \cdot 0}$–$10^{8 \cdot 5}LD_{50}$. A mouse is therefore capable of adsorbing 50–150 times the amount of virus contained in a saturating inoculum.

It is shown that the virus which disappears from the blood during adsorption is no longer detectable anywhere in the mouse.

The median death-time in inoculated mice is inversely proportional to the logarithm of the infective dose. However small the inoculum, nearly all deaths occur within a hundred hours of inoculation. An occasional death after 8–10 days is shown to be due to infection resulting from cannibalism.

However large the inoculum, death does not occur until a single cycle of growth is completed. A mouse may die, probably as a result of virus multiplication, as early as 6 hr. after an extremely large inoculum.

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RIFT VALLEY FEVER VIRUS IN MICE. IV. INCOMPLETE VIRUS; ITS PRODUCTION AND PROPERTIES

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EARLY in these studies it was discovered that mice died with different blood titres. In routine passages 10^{-1} dilutions of infective serum were inoculated, and although occasionally a serum pool was obtained which had a titre of $10^{8.5}$ LD_{50}/0·03 ml. or greater, titres of $10^{7.0}$–$10^{8.0}$ were more common, and levels as low as $10^{5.0}$ were encountered. Stock sera whose titre was found to be $10^{7.0}$ or greater were selected for use in experiments, but mice inoculated with these sera in concentrated form might die with low or with high blood titres. As a result titration end-points were sometimes missed in growth curve experiments, because peak titres were unpredictable. This difficulty persisted during much of the work described in previous papers.

On one occasion a pooled blood titre of $10^{8.8}$ occurred in mice sick after an inoculum of stock infective serum which had been diluted 10^{-4}, instead of 10^{-1} as was usual. It was thought possible that this uncommonly high titre occurred because of the relatively small amount of virus contained in the 10^{-4} diluted inoculum. It is known that when influenza virus grows in the allantoic cavity, the final infective titre depends on the amount of virus inoculated. Von Magnus (1951*a*) showed that when undiluted allantoic fluid was serially passaged, peak infective titres fell, whereas they were regularly high during serial passages of 10^{-6} diluted material. Peak infective titres were shown to be low because so-called "incomplete" virus had been produced. It was these and subsequent studies on "incomplete" influenza virus which prompted the investigations described in this paper.

MATERIALS AND METHODS

Virus.—As in Paper I. The standard diluent was pH 7·4 Sørensen-buffered 0·5 per cent bovine plasma albumin.

Mice.—As described in Paper I.

Routine titrations.—As described in Paper I. Organ titres expressed as $\log_{10}$ LD_{50}/0·03 ml. tissue.

Carcass titrations.—As described in Paper II.

Neutralisation tests.—As described in Paper I.

RESULTS

Serial passage of infective serum at various dilutions

It was thought that the serial passage of infective serum at various dilutions would show directly whether peak blood titres were low when there were large amounts of virus in the inoculum. Virus was serially passaged using undiluted,

10^{-1}, 10^{-4}, 10^{-6} and finally 10^{-8} diluted infective serum. At each passage pooled serum from 3–6 sick or recently dead mice was diluted to the appropriate extent and inoculated immediately or after storage up to 24 hr. at −20°. The titre was as a rule determined directly by titration, but it was occasionally very roughly calculated from the observed median death time (see below). Independent passages were sometimes made from a given serum either concurrently or after storage of the serum at −20°. It was shown in a preliminary experiment that the ability of high titre sera to produce high titres when inoculated undiluted and 10^{-2} diluted (see below) was unaffected by storage up to five weeks at −20°.

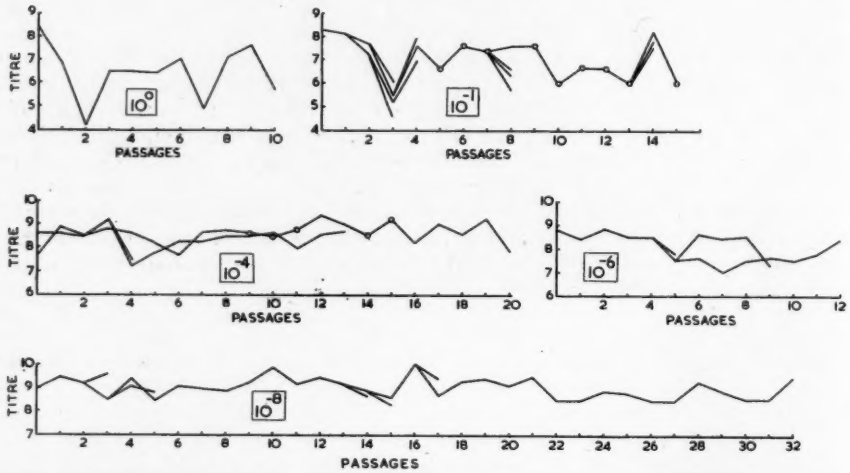

FIG. 1.—Continuous serial passages of R.V.F. virus infective serum at various dilutions. All inoculations were intracerebral. Each point on each passage line is the titre of pooled serum from 3–5 sick or dead mice which had been inoculated with the appropriate dilution of the preceding serum in the passage series. The serum titre was usually determined directly by titration, although at the points indicated by circles it was roughly calculated from the death-time in inoculated mice. The titre of passage No. 0 is in each case the titre of the serum used to start the series. Distinct passage lines, which sometimes originate from the same infective serum, are shown as distinct lines. Titres in $\log_{10}$ $LD_{50}/0{\cdot}03$ ml. serum. All titres shown on the same scale.

Results are shown graphically in Fig. 1. It can be seen that in the undiluted and 10^{-1} diluted serial passages titres fluctuate, and fall as low as $10^{4{\cdot}2}$, with recoveries which rarely reach the initial levels. Sera passaged at 10^{-4} and 10^{-6} in general maintained high titres, but even here there were falls to titres of less than $10^{8{\cdot}0}$, which could be produced on more than one occasion from the same serum. In the 10^{-8} passage series, however, titres never fell below $10^{8{\cdot}5}$, although 32 consecutive passages were made.

Often with the concentrated inocula (undiluted and 10^{-1} diluted passage series), a given serum, although itself of high titre, would repeatedly produce low titres in inoculated mice, on separate occasions. Again, a low titre serum might repeatedly produce high titres. There is therefore something about an infective serum which makes it give rise to low or high titres when inoculated in concentrated

form. Even two sera of the same infectivity titre may behave differently, one producing high and one producing low titres at the next passage. Clearly there is something in addition to the mere infectivity titre that plays a part in these phenomena.

Characterisation of sera by titres produced in individual mice.—All titres in the passage series were determined for serum pools, and it was thought that individual mouse titres might vary while the pooled titre, as measured on a log scale, reflected that of the highest contributor. A series of individual serum titrations were therefore made from mice when sick following inoculation with the appropriate dilutions of 10^{-1}, 10^{-4} and 10^{-8} passaged sera. The results (Table I) show that mice given the 10^{-1} serum passaged at 10^{-1} had low but variable titres; the titre of these when pooled would have been $10^{6\cdot4}$. Thus when peak titres following a given inoculum are low they are very variable in individual mice, as measured on a log scale. Since a random sample of these is taken when 3–6 mice are bled, some irregularity in the level of low titres is to be expected. Mice given one of the 10^{-4} sera at 10^{-4} had high titres, but there were low and variable titres with the other 10^{-4} serum tested. All except one of 34 mice given 10^{-8} serum passaged at 10^{-8}, however, had titres greater than $10^{8\cdot0}$, and only 7 of them were lower than $10^{8\cdot5}$. It is concluded, first, that sera with low titre-producing power may occur in the 10^{-4} passage series, and second, that the titres produced following 10^{-8} diluted inoculations of 10^{-8} passaged serum, are high in all individual mice.

TABLE I.—*Peak Blood Virus Titres in Individual Mice after Inoculation with the Appropriate Dilution of* 10^{-1}, 10^{-4}, *and* 10^{-8} *Passaged Sera.*

Inoculum.	Titre of serum inoculated.	Titres in individual mice.											
10^{-1} passaged serum diluted 10^{-1}	7·8	7·1	6·8	6·7	6·5	6·0	5·4	5·4	5·2	5·0	4·8	<4·0	—
10^{-4} passaged serum diluted 10^{-4}	8·8	8·6	8·6	8·6	8·4	8·4	7·8	7·8	7·8	7·8	—	—	—
	9·2	7·8	>7·0	>7·0	>7·0	6·5	6·0	5·6	—	—	—	—	—
10^{-8} passaged serum diluted 10^{-8}	8·6	9·5	9·0	9·0	8·7	8·6	8·5	8·5	8·3	8·2	8·0	—	—
	8·7	9·6	9·1	9·0	9·0	9·0	8·7	8·7	8·6	8·6	8·2	8·1	7·5
	9·5	9·5	9·5	9·1	9·1	9·1	8·7	8·6	8·6	8·5	8·5	8·5	8·4

The titres of the 34 mice inoculated with 10^{-8} diluted serum showed a variation of $10^{2\cdot1}$, or $10^{1\cdot6}$ if the lowest titre is not counted, and it was shown in Paper I that the experimental titration error was not usually greater than $10^{0\cdot5}$. The arithmetical variation in individual titres, therefore, is considerable even when high titre-producing sera are inoculated. The differences between individual titres presumably reflect differences in the ability of individual mice to support virus growth.

Characterisation of sera by the titres produced on inoculation at various dilutions.—It had been found on many occasions that a serum which produced low peak titres when inoculated in concentrated form would produce high titres when considerably diluted. For instance, in the undiluted intracerebral passage series (Fig. 1), two sera of titres $10^{6\cdot8}$ and $10^{4\cdot2}$, which produced as low or lower

titres when passaged undiluted, nevertheless produced titres of $10^{8\cdot5}$ and $10^{8\cdot4}$ when passaged at limiting ($10^{-5\cdot5}$ and $10^{-3\cdot5}$) dilutions.

Rather low titres occurred in the 10^{-4} passage series, and it was thought that any low titre-producing power of these sera might be more clearly demonstrated in the following way. Sera were diluted in serial tenfold steps, and each dilution inoculated intracerebrally into a box of 6 mice. Three to 6 mice from each box were bled when sick or recently dead, and their pooled blood titrated. For comparison, a few sera from the 10^{-8} passage series were dealt with similarly. Results are shown in Table II. It is seen that none of the 10^{-4} passaged sera tested produced high titres with all the dilutions inoculated. Each of the 10^{-8} passaged sera, however, gave high titres, whichever dilution was inoculated.

TABLE II.—*Titres Produced by Sera on Inoculation at Various Dilutions.*

Serum inoculated.			Dilution inoculated.								
			10^{0}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}
10^{-4} passaged.	Titre	$10^{8\cdot5}$.	7·1	7·5	5·8	7·6	8·8	7·9	—	—	—
10^{-4} ,,	,,	$10^{8\cdot8}$.	7·6	7·1	6·4	6·7	7·8	8·2	—	—	—
10^{-4} ,,	,,	$10^{7\cdot8}$.	8·4	6·8	6·7	8·5	9·0	8·9	—	—	—
10^{-8} ,,	,,	$10^{9\cdot2}$.	9·4	9·2	8·9	8·8	8·7	9·2	—	—	9·5
10^{-8} ,,	,,	$10^{8\cdot9}$.	9·6	9·5	8·4	9·0	9·4	9·4	8·2	8·6	8·4

Although two of the 10^{-4} passaged sera tested in this experiment were themselves of high titre ($10^{8\cdot5}$ and $10^{8\cdot8}$), they thus possessed low titre-producing power, as detected in this way. They produced titres as high as $10^{8\cdot5}$ and $10^{8\cdot8}$ when inoculated at 10^{-4} or 10^{-5}, but these were from serum pools, and some of the individual mice may nevertheless have had low titres (see above).

It is worthy of note that mice given 10^{-2} diluted low titre-producing sera had lower titres than those given undiluted sera.

Post-mortem incubation does not account for the low titre-producing power of sera.—In the serial passages mice were often bled when dead, and it was sometimes difficult to judge how recently they had died unless the corpse was still soft and warm. An experiment was therefore performed to see whether blood developed the capacity to produce low titres when it remained in the body for long after death. Blood from the tail of a mouse sick following a 10^{-4} diluted inoculum of high titre serum was obtained, titrated, and then inoculated at 10^{-1} to 6 mice, whose pooled blood was in turn titrated when they were sick. Tenfold diluted blood was inoculated because it was thought that this, the most concentrated inoculum readily obtainable from the sick mouse, would be the most sensitive test of any low titre-producing power. After the mouse had been dead 3 hr. blood was again obtained, titrated, and passaged at 10^{-1} and titrated. In another experiment blood was taken from a mouse sick following a 10^{-8} diluted inoculum of high titre serum, and treated in the same way. The mouse died $2\frac{1}{2}$ hr. later, and 5 hr. and 20 hr. after death blood was again taken and dealt with similarly. The results (Table III) show that prolonged post-mortem incubation at room temperature (22–26°) affects neither the blood titre, nor the ability of the blood to produce low titres.

Carcass titres are low when blood titres are low.—In the serial passage experiments blood or serum was titrated. On several other occasions livers, and once an entire carcass (see Paper II for methods), were titrated in sick mice whose

TABLE III.—*The Effect of Post-mortem Incubation on Blood Titres and on the Capacity of Blood to Produce Low Titres.*

	Blood titre.	Results of inoculation of each blood, tenfold diluted, into four mice.	
		Time at which half these mice were dead.	Titre of pooled blood from the four inoculated mice.
Sick mouse A	8·9	17 hr.	7·4
Mouse A dead 3 hr.	8·7	18 „	7·7
Sick mouse B	8·9	18 „	9·5
Mouse B dead 5 hr.	8·8	17 „	9·2
„ B „ 20 hr.	8·5	21 „	8·7

blood titre was low. Table IV summarises these experiments, and it can be seen that liver titres and the carcass titre were also low. The high titres which occurred in mice sick from a dilute inoculum of high titre serum are shown in the Table for comparison. Thus, low titres are produced in the entire mouse, and not just in the blood, when concentrated inocula are passaged.

Table IV also shows that although the liver titre is low in low-titre-mice, it is often tenfold or even a hundredfold higher than the blood titre. This is not usually the case (see Paper I), and suggests, first, that virus is coming from the liver, and, second, that there may be some delay in its release when blood titres are low.

TABLE IV.—*Organ and Carcass Titres in Individual Mice in which Peak Blood Titres were Low.*

Inoculated blood.		Incubation period (hr.)	Blood titre.	Liver titre.	Carcass virus content.*
$\log_{10}$ titre.	Amount given.				
3·7	0·1 ml. I.V.	96	4·4	6·0†	—
5·9	„	13½	3·6	4·5	—
5·9	„	14	<3·0	3·7	—
5·9	„	14	<3·0	4·2	—
6·1	„	14	4·3	>5·0	—
6·8	„	43	4·3	4·5	5·8 (6·0)
6·8	„	46	3·2	4·1	—
7·2	0·03 ml. I.C. at 10^{-1}	22½	7·0	7·5	—
7·2	0·03 ml. I.C. at 10^{-1}	24½	5·2	7·5	—
8·2	0·03 ml. I.C. at 10^{-4}	33	8·4	8·2	10·0 (9·9)

For comparison pooled titres in two mice with high blood titres are shown at the bottom of the Table.

* Carcass virus content, directly determined, in $\log_{10}$ LD_{50}. Titres per whole mouse blood, calculated from the blood titration, are included in brackets.

† The brain titre in this mouse was found to be $10^{2\cdot0}$.

Dose-incubation period relation of low titre sera

In Paper III of this series, the median death time in mice was shown to be proportional to the logarithm of the dose of virus inoculated. A high titre 10^{-4} passaged serum was used in that experiment, and it was frequently noticed that

low titre sera gave much later death-times when a given infective dose was inoculated. A more detailed study of the incubation periods (as represented by the death-times) of these low titre sera inocula was therefore made.

In these experiments two sera (of titres $10^{3\cdot2}$ and $10^{5\cdot2}$) from the undiluted intravenous passage series (see below), a serum of titre $10^{7\cdot4}$ from a 10^{-1} passage series, and a high titre serum ($10^{8\cdot1}$) from a 10^{-4} passage series, were studied. They were diluted in serial tenfold or $10^{0\cdot5}$-fold steps, and each dilution inoculated into 15–18 mice. Large inocula, of a volume greater than 0·03 ml., were given intravenously to a smaller number of mice. Death times were recorded and the median value calculated (Fig. 2). For the $10^{7\cdot4}$ titre serum the mean instead of

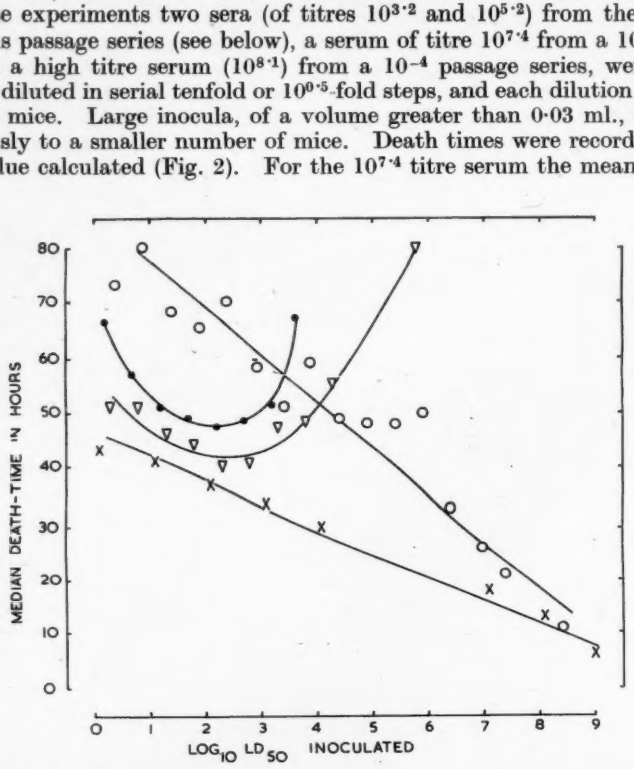

FIG. 2.—Relation between dose and incubation period, for sera of titres $10^{8\cdot1}$ (crosses), $10^{7\cdot4}$ (circles), $10^{5\cdot3}$ (triangles) and $10^{3\cdot2}$ (dots). Each serum dilution was inoculated into 10–15 mice, except in the case of the largest inocula, which were given to smaller numbers. Death-times were recorded, and the median death-time (mean death-time for the serum of titre $10^{7\cdot4}$) calculated.

the median death-time had been calculated and points are irregular, but the relationship is probably a straight line one. Large doses of this serum gave death-times as early as those produced by the high titre serum, but all smaller doses resulted in much later death times. This difference was apparent when as few as 1–10 LD_{50} were inoculated. Moreover, there was a much greater scatter in individual death-times following inoculations of this serum, and even if the occasional unusually late death was not counted, mice receiving a given inoculum died over the course of 20–50 hr. When, however, any dilution of the high titre serum was inoculated, and the occasional late death again not counted, mice were found to die within 10 hr. of one another. This high titre serum, from a

10^{-4} passage series, gave median death-times as short as did any serum encountered in the 10^{-8} passage series. Thus, the most rapidly lethal virus material obtainable was probably present in this serum.

The very low titre sera obtained in the undiluted intravenous passage series gave curves of a different shape. When small infective doses were inoculated these sera also gave a later median death-time as well as a greater scatter in individual death-times than did the same infective dose of the high titre serum. When larger amounts were inoculated, however, death-times were very much delayed. It was after large inocula of one of these low titre sera that mice died with even lower blood titres, others died after 6–12 days with virus in the brain but not in the liver, and a few survived and became immune. To see whether these much prolonged incubation periods could be shortened if enough infective virus was present in the inoculum, 0·1 ml. of one of these low titre sera was given intravenously to mice, together with an equal volume of high titre serum containing about $10^{9\cdot5}$ LD_{50}. Incubation periods were then as short as when the high titre serum was administered alone. Thus, although large doses of low titre sera may give prolonged incubation periods, the effect can be overcome if enough infective virus is inoculated. Perhaps this is why the dose-incubation period curve for the 10^{-1} passaged serum (Fig. 2) approaches the high titre serum curve as the infective dose becomes very large.

Demonstration of non-infective interfering agent in low titre-producing sera.

The results of the serial passage experiments show that when large inocula of low titre-producing serum are inoculated, something, presumably in the inoculated serum, prevents high peak titres being reached. The dose-incubation period experiments indicate that something in such serum also prolongs the incubation period. These effects were directly demonstrated in the following growth curve experiment. A 10^{-4} passaged high titre serum was diluted 10-fold and inoculated intracerebrally in 4 adult mice. Two hours later half the mice were given 0·2 ml. of a low titre-producing serum intravenously. The titre of this serum was $10^{5\cdot5}$, so that it was a relatively minute extra infective dose, and as low or lower titres were produced when it was inoculated undiluted. Growth curves were constructed for both groups of mice from the titration of pooled tail blood samples at fixed time intervals. Results (Fig. 3) show that the low titre serum markedly depressed the infective virus yield and in addition prolonged the incubation period, compared with the yield and incubation period when high titre serum alone was given. The fall in titre during the 22 hr. before mice began to die might be a result of thermal inactivation of virus at the body temperature of the mice. In Paper II it was shown that a similar 10-fold fall in titre occurred when infective serum was incubated at 37·5° for 24 hr.

To make sure that the titre of a high titre serum is not depressed when it is merely mixed with a low titre serum, another experiment was performed. A low titre serum when mixed with an equal quantity of standard diluent had a titre of $10^{5\cdot5}$. A high titre serum mixed similarly had a titre of $10^{8\cdot2}$. When the two sera were mixed in equal quantities the titre was $10^{8\cdot3}$, so it appears that low titre sera cannot by their very presence depress the titration end-point of high titre sera.

Low titre sera therefore contain something which both depresses the final

virus yield and prolongs the incubation period when very large quantities are inoculated into mice following relatively small quantities of high titre serum.

Blood virus growth curves were then constructed, using a low titre-producing serum by itself as inoculum. A certain 10^{-1} passaged serum had repeatedly produced titres of $10^{6 \cdot 0}$ or less on different occasions. Six mice were inoculated

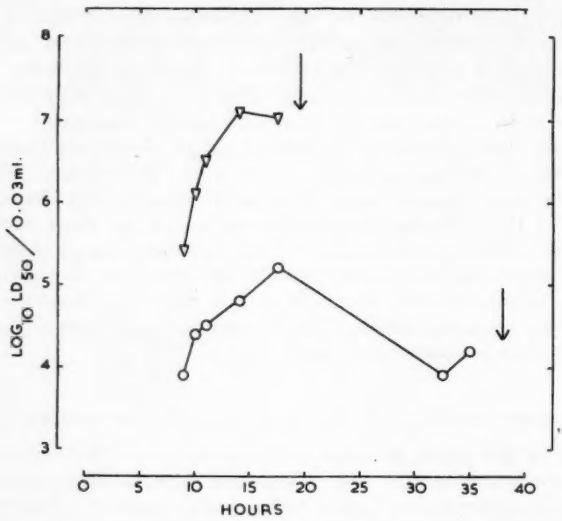

FIG. 3.—The depression of the peak titre and lengthening of the incubation period by large doses of low titre serum. Each growth is for virus in the pooled blood samples from two inoculated mice. One group received $10^{7 \cdot 0}$ LD_{50} of a high titre ($10^{8 \cdot 0}$) serum intracerebrally (triangles), and the other group (circles) an identical inoculum followed two hours later by 0·2 ml. of a low titre serum intravenously. Arrows above the growth curves indicate the time at which mice began to sicken and die.

with a 10^{-1} dilution of this serum, and a growth curve (Fig. 4) was constructed from repeated pooled samples of their tail blood. The first cycle of growth (see Paper II) is visible, but the titre did not continue to increase exponentially, and stayed at $10^{5 \cdot 5}$–$10^{6 \cdot 0}$ for about 20 hr., when the mice began to die. The shape of the curve is very similar to that obtained in the interference experiment (Fig. 3), suggesting that an interfering agent contained in the low titre-producing serum was again depressing peak titres and prolonging the incubation period. Another growth curve, where a low titre was produced following a 10^{-1} inoculum, is included in Fig. 4, together with the type of two-step growth curve where a peak titre of $10^{8 \cdot 5}$ is reached (see Paper II).

Antibody-binding power of low titre-producing sera diluted beyond their infectivity end-point

A high titre 10^{-4} passaged serum was diluted $10^{-5 \cdot 5}$ (to contain about $10^{3 \cdot 0}$ LD_{50}/0·03 ml.) and added to (*a*) the same volume of serial tenfold dilutions of the author's R.V.F. virus immune serum, each immune serum dilution having been

mixed 30 min. earlier with the same volume of buffered 0·5 per cent bovine plasma albumin and (*b*) the same volume of serial 10-fold dilutions of this immune serum, each serum dilution having been mixed 30 min. earlier with the same volume of a low titre serum diluted beyond its end-point. The low titre serum, of titre $10^{4\cdot2}$, was diluted $10^{-5\cdot0}$, and did not then kill mice when inoculated by itself. The contents of each tube were inoculated (0·03 ml.) intraperitoneally into five adult mice. Considered as an immune serum titration, the Reed-Muench end-point for (*a*) was $10^{3\cdot9}$ and for (*b*) $10^{3\cdot2}$.

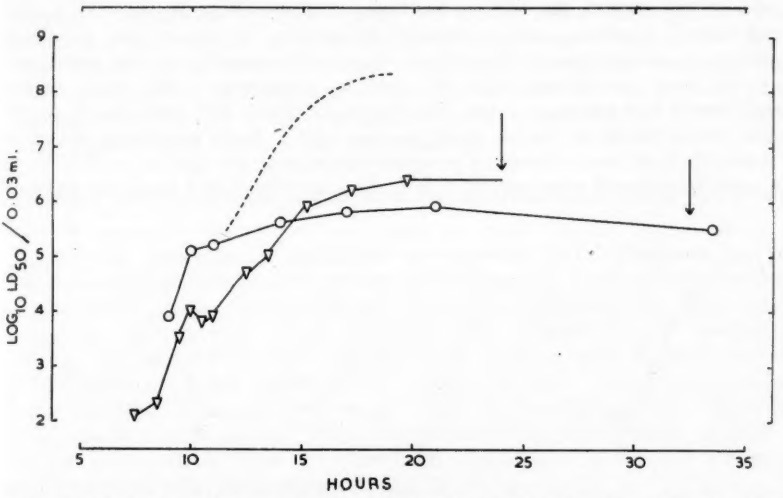

FIG. 4.—Blood virus growth curves in mice following inocula which produced low peak titres. The points on each curve represent the pooled blood samples from the same six mice, taken at fixed time intervals. Inoculations were $10^{6\cdot3}$ LD_{50} of serum of titre $10^{7\cdot3}$ (circles), and $10^{7\cdot3}$ LD_{50} of serum of titre $10^{8\cdot3}$ (triangles). The shape of the second part of the type of two-step growth curve when high titres are produced, is included as a dotted line. (See Paper II.)

To confirm that there was a real difference, another low titre serum (titre $10^{5\cdot4}$) was used, and the titrations were made more accurate by using serial $10^{0\cdot5}$-fold dilutions of immune serum, and inoculating the contents of each tube into 8 mice. Once again, mice given the diluted low titre serum alone did not die. The immune serum end-point for (*a*) was $10^{6\cdot1}$, and for (*b*) $10^{4\cdot8}$. It would appear, therefore, that the antibody titre of an immune serum is reduced when mixed with a low titre infective serum diluted beyond its end-point. Both the low titre sera used produced low titres when inoculated undiluted.

Serial passage of undiluted blood intravenously, the survival of mice, and the localisation of virus in the brain

Undiluted infective blood was passaged intravenously, to see whether, with the larger inocula which could thus be given, even greater falls in titre would occur. It was shown in Paper I of this series that the response to intravenously

and to intracerebrally inoculated R.V.F. virus was the same, the latter acting as a small intravenous inoculum. The blood used to start the series had previously had four 10^{-4} passages in mice, was of high titre and high titre producing. Twelve mice were inoculated intravenously with 0·1 ml. of this blood, and when they were sick or recently dead blood was taken from the thorax into a tuberculin syringe, of which the needle contained a trace of heparin. The blood was pooled, titrated, and inoculated intravenously in the mice of the next passage. Since a large number of mice were used to start the series, there was enough blood available at each passage to inoculate at least six mice. Any blood left over was centrifuged, and the serum stored at −20°. Successive titres (Fig. 5) showed a regular "switchback" pattern, and a similar succession of titres was obtained in independent passage lines. High titres were maintained for a few passages and then there were precipitous falls, followed by recoveries; this cycle of events recurred every 4–5 passages. On occasion mice given the sera which produced minimal titres failed to die or even become sick. Such surviving mice, when bled a month later, were found to possess neutralising antibodies to R.V.F. virus. Of 16 mice inoculated with serum 3 (Fig. 5) 5 survived and acquired antibodies.

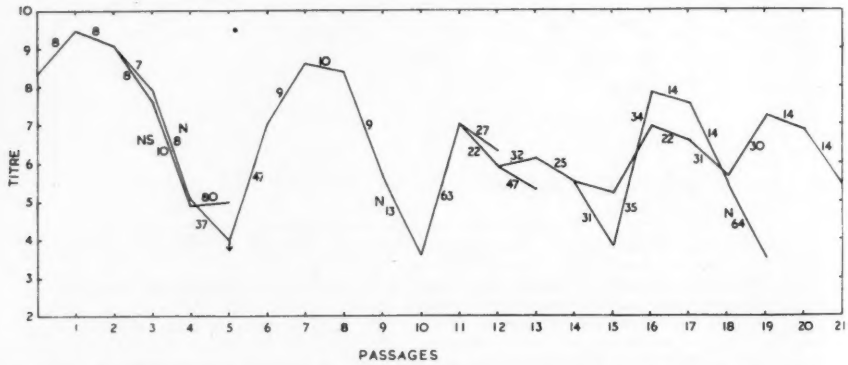

FIG. 5.—Serial passages using undiluted blood inoculated intravenously. Each point obtained by titrating pooled blood from at least 6 sick or dead mice, except in the case of one or two very low titre bloods where smaller numbers were bled, because the rest either died very late or survived (see text). The time in hours at which mice were bled is indicated against the line leading to the titre of the blood obtained. At least one mouse was dead, and the rest sick, at the time of bleeding. When any mice died late "neuro"-deaths or survived the fact is indicated by "N" or "S" respectively against the incubation period of those who were bled. Titres in $\log_{10}$ LD_{50}/0·03 ml. blood.

The phenomenon was noticed again when 2 mice survived and became immune during the course of another intravenous passage series using undiluted blood.

When mice were inoculated with certain sera of titre $10^{5.0-6.0}$, they died with even lower blood titres, and did so after lengthy incubation periods (see, for example, Fig. 2). In Paper III R.V.F. virus was shown to kill inoculated mice within 5 days, however small the inoculum, but after these low titre sera inoculations some mice died after as long as 6–12 days. Their seitz-filtered liver suspensions, however, failed to kill inoculated mice, and since a few of them became paralysed or sick the day before death occurred, seitz-filtered brain suspensions were also

tested, and found to kill mice in 2–3 days. Thenceforth livers and brains were tested, and it was discovered that each of 8 mice sick or dead 6–12 days after large inoculations of four different $10^{5\cdot0-6\cdot0}$ titre bloods, had virus in the brain but not in the liver. Such virus was neutralised by R.V.F. virus immune serum, and behaved in a viscerotropic way, killing adult mice 2–3 days after intraperitoneal inoculation and producing characteristic changes in the liver.

Thus, when some of the lower titre bloods in this passage series were given in large doses an occasional mouse survived and became immune, and, in those dying very late after a prolonged period of sickness, virus was present in the brain but not in the liver. Other equally low titre bloods occurring in the passage series did not give rise to these phenomena, which were restricted to the very low titre bloods which contained large amounts of interfering material, in that they produced lengthy incubation periods and even lower titres at the next passage. The phenomena did not occur, for instance, with blood of titre $10^{3\cdot0}$, and such blood produced higher titres at the next passage.

Unexpectedly short incubation periods following maximal doses of low titre-producing sera

On several occasions in undiluted intravenous serial passages incubation periods following inoculations of low titre-producing sera, far from being prolonged, were very short, even when compared with those following the same infective dose of high titre serum. These low titre sera contained large quantities of interfering material, for they not only gave rise to even lower titres in inoculated mice, but some produced late " neuro "-deaths as well. Death-times following large intravenous inoculations of two of these sera, compared with the shortest death-times ever encountered with similar infective inoculations of high titre serum, are included in Table V. The high titre serum is the one whose dose-incubation period relation is shown in Fig. 2.

TABLE V.—*Death-times in Mice Inoculated with Large Doses of Certain Low Titre Sera.*

	Inoculum.	Number mice inoculated.	Median death-time (hr.).	Shortest death-time in any individual mouse (hr.).
Low titre sera	$10^{6\cdot4}$ LD_{50} of $10^{5\cdot9}$ titre serum	16	17	11
	$10^{6\cdot2}$ LD_{50} of $10^{5\cdot7}$ „ „	7	13	9
High titre sera	$10^{7\cdot1}$ LD_{50} of $10^{8\cdot1}$ titre serum	9	18	15
	$10^{6\cdot2}$ LD_{50} of $10^{8\cdot1}$ „ „	—	20	—

Death-times for high titre, quick killing sera are shown for comparison. Exactly equivalent infective doses were not inoculated: in the first, the nearest largest dose inoculated is shown and in the second, the median value was read off on the high titre serum dose-incubation period curve.

DISCUSSION

It has been shown that when R.V.F. virus is passaged in concentrated form sera with low infectivity titres are produced, which on inoculation give prolonged incubation periods. It was demonstrated directly that this was due to an interfering agent which was present in low titre-producing sera. The effect

could not be accounted for by post-mortem incubation of blood. The phenomenon is thus similar to that described for influenza virus in the allantoic cavity (von Magnus, 1951*a*) and de-embryonated egg (Bernkopf, 1950). The infectivity titre of the allantoic fluid was shown to fall when large inocula were passaged, although the haemagglutinin titre was, initially, unchanged. It was concluded that so-called " incomplete " influenza virus particles had been produced, which haemagglutinated but which were not infective. It was later shown that such particles also combined with antibody, fixed complement, interfered with the growth of infective virus, were adsorbed on to and eluted from red blood cells, and were antigenic.

Incomplete R.V.F. virus can therefore be produced in the same way as incomplete influenza virus. It has been shown that a non-infective agent in low titre-producing R.V.F. sera will combine with antibody, and, moreover, that large doses of low titre-producing sera may be antigenic, if they are not lethal. It is concluded that incomplete R.V.F. virus not only depresses peak titres and prolongs incubation periods, but also combines with specific antibody and is antigenic.

The effect of incomplete influenza virus on the incubation period is not demonstrable in the chick embryo, but von Magnus (1951*b*) showed that low titre allantoic fluids (produced by undiluted serial passage) inoculated intranasally in mice resulted in longer incubation periods, and fewer deaths and lesions, than did high titre (10^{-6} serially passaged) virus.

The prolongation of the incubation period when the inoculum contains large quantities of incomplete virus might be attributed to interference, or the "blockage " of susceptible cells by incomplete virus particles. If, however, the majority of susceptible cells have supported virus growth by the time of death, any blocking effect must by then have been overcome, allowing infective particles to grow in cells. Moreover, the " blocking " effect would presumably disappear on dilution, yet low titre sera, even when diluted 10^{-7} (see Fig. 2) have longer incubation periods than do equivalent inocula of high titre sera. To explain this latter phenomenon it seems necessary to postulate that the virus particles in low titre serum differ in that they are either not capable of such rapid growth, or are not so rapidly lethal when they grow, as are the particles in high titre serum. This means that there may be degrees of " incompleteness ".

The unusual shape of the dose-incubation period curve of very low titre sera (Fig. 2) is difficult to account for. As larger amounts of the sera were inoculated the incubation period became progressively more prolonged. It was shown that the effect could be overcome if enough infective virus was present in the inoculum. Presumably larger inocula contained larger amounts of the agent which prolonged the incubation period, but not enough infective virus to overcome the effect.

The infectivity titre of a serum does not by itself give reliable information as to the incomplete virus content. For instance, in the 10^{-4} serial passages sera with titres as high as $10^{8.5}$ or $10^{8.8}$ (Table II) may produce low pooled titres when inoculated in concentrated form, and a low titre in an occasional mouse even when inoculated at 10^{-4} (Table I). Again, the 10^{-4} passaged serum in Fig. 2 gives much the same dose-incubation period curve as any 10^{-8} passaged serum. The 10^{-4} passaged serum studied in Paper III of this series, however, gave considerably longer incubation periods for given infective doses. One concludes that this latter serum, in spite of its higher titre ($10^{9.0}$), contained incomplete, or slow

growing, particles. The various passage series in Fig. 1 and 5 were started with different 10^{-4} passaged sera, which contained different quantities of incomplete virus. If the starting serum in the undiluted intracerebral series, for instance, contained more incomplete virus than in the undiluted intravenous series the more rapid fall in successive titres is explained. Incomplete virus is probably produced even in the 10^{-6} passage series (Fig. 1), for low titres occurred here as they did in the 10^{-4} series. If an infective serum produces high titres whatever the dilution inoculated, it probably contains little or no incomplete virus. Such serum is obtained when virus is serially passaged using a dilution factor of 10^{-8}. The $\log_{10}$ infectivity titre, therefore, is an unreliable guide to the incomplete virus content of a serum. Even if half the particles are incomplete the titre is only reduced by $10^{0 \cdot 3}$, and this is within the range of experimental titration error (see Paper I).

In the undiluted serial passages large doses of low titre sera frequently produced low titres, whereas the same infective dose of high titre sera (Table II) produced high titres. This is presumably because the non-infective, interfering particles in low titre sera contribute to the low titre-producing effect, as has also been postulated for incomplete influenza virus by von Magnus (1954). If the virus which is serially passaged in concentrated form is 100 per cent complete to start with it is logical to assume that incomplete virus is produced even at the first passage, although the $\log_{10}$ titre is not detectably lowered. On further passage the infective titre falls progressively (incomplete virus accumulates), because the large quantity of incomplete virus produced together with infective virus still ensures that a very large particle dose is inoculated. At some stage, however, either the total number of particles produced diminishes or, alternatively, many of the particles become too incomplete even to interfere. At this stage large inocula of low titre sera contain much less interfering material, so that titres rise again. Such a sequence of events explains the " switchback'" effect in the undiluted intravenous serial passages (Fig. 5), where there are slow falls in titre followed by relatively rapid recoveries. If interfering virus accumulated indefinitely such recoveries would, of course, be impossible. It follows that sera containing the largest amounts of interfering material are those which produce the lowest titres, *i.e.*, sera nos. 3, 4, 9, 14, and 18 in Fig. 5. It was these sera, moreover, which produced the late " neuro " deaths and survivals with immunity, additional evidence that maximum amounts of interfering material were present.

The interfering property of incomplete virus may account for the mice in the intravenous passage series which died after 7–12 days with virus in the brain but not in the liver. Visceral cells may have been occupied by incomplete particles, but if an infective particle somehow chanced to reach an extravascular site in the brain it would grow if it was potentially, or from the start, neurotropic. R.V.F. virus recovered from the brains of mice which died very late was viscerotropic, and probably viscerotropic R.V.F. virus is capable of slow growth in the brain, although it rarely gets a chance to do so because it kills the host too soon.

The occasional mice which survived and became immune after large doses of low titre serum probably did so as a result of the interfering and antigenic properties of the incomplete particles. It is assumed that infective virus could not have multiplied and itself produced antibodies without causing sickness or death. Either incomplete virus multiplied in cells to produce more incomplete virus, or, more probably, immunity was produced by the sheer antigenic bulk of

the inoculum. Each immune survivor had received a dose of infective virus which, on dilution, would kill hundreds of thousands of mice, but the enormous amounts of interfering material present prevented the growth of infective particles.

In the intravenous passage series (Fig. 5) mice sicken and die with blood titres as low as 10^3 to 10^5, that is to say, when virtually all the virus produced is incomplete. If death is a result of cell damage it seems that this damage is just as extensive when incomplete particles are produced. Perhaps, therefore, there is no very great difference in the behaviour of virus during most of its growth in the cell, whether complete or incomplete particles are yielded. This may mean that incomplete virus results from some defect operating fairly late in intracellular development.

Low titre sera sometimes gave unexpectedly short incubation periods when large amounts were inoculated (Table V). These low titre sera produced even lower titres at the next passage, and some produced "neuro" deaths as well. They therefore contained maximal amounts of interfering material and one would expect to see prolonged incubation periods when large doses were inoculated (Fig. 2). The fact that incubation periods were even shorter than warranted by the amount of infective virus administered is not easy to explain. Conceivably some sort of " multiplicity reactivation " took place, so that complete particles were produced in cells from the pooled fragments of incomplete ones.

Incomplete virus formation has been thoroughly recorded and studied only for influenza virus. The incomplete virus type of phenomenon, however, has been reported on occasions. For instance, yellow fever investigators have recorded that monkeys and galagos given small inocula usually attain higher blood titres than those given large inocula (Smithburn and Haddow, 1949; Smithburn, 1949).

It should not be forgotten how artificial the circumstances are when incomplete virus is produced. By means of a syringe or pipette virus is confronted with a situation it has never before encountered. Nature's inocula, whether by insect-bite or infective droplet, are usually much smaller, and it is not surprising that the highly efficient system of virus growth which has always followed such inocula should break down under unprecedented laboratory circumstances. Nevertheless, from an analysis of the growth disorders which follow these large inocula, some insight into the normal mechanism and requirements of virus growth may eventually be obtained.

SUMMARY

Low R.V.F.virus infectivity titres are produced when large inocula are serially passaged, and high titres when small inocula are passaged. This is explained by the production of an incomplete, non-infective form of virus.

The incomplete virus present in low titre-producing sera is shown to interfere with the visceral growth of infective virus, first, by prolonging the incubation period and second, by reducing the peak infectivity titre attained.

Very large quantities of incomplete virus may interfere with visceral multiplication to such an extent that mice survive, or if they do die, some of them do so as a result of virus growth in the brain, not in the liver. Since the surviving mice develop specific neutralising antibodies, it is concluded that incomplete virus is antigenically active.

Incomplete virus combines with specific neutralising antibody.

It is shown that, by itself, the $\log_{10}$ infectivity titre of a serum is an unreliable guide to the incomplete virus content. Little or no incomplete virus is produced when serum is serially passaged using a dilution factor of 10^{-8}.

The suggestion that a non-interfering type of incomplete particle is eventually produced during the serial passage of large inocula explains the regular reappearance of high titres.

An inoculum may be lethal even when almost all the virus yielded is incomplete.

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THE CHEMICAL BASIS OF THE VIRULENCE OF *BACILLUS ANTHRACIS*. VI: AN EXTRACELLULAR IMMUNISING AGGRESSIN ISOLATED FROM EXUDATES OF INFECTED GUINEA-PIGS

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THE literature (Smith and Keppie, 1955) indicates that at least two factors are involved in the aggressive action of *Bacillus anthracis*; one is connected with the capsule and the other is an extracellular product. Bail (1904) showed that injection of anthrax exudates into animals would immunise them against subsequent infection with *B. anthracis* and he speculated that the extracellular aggressin and the protective antigen were the same substance. In attempts to identify compounds produced by *B. anthracis*, Cromartie, Bloom and Watson (1947), Cromartie, Watson, Bloom and Heckly (1947), Watson, Cromartie, Bloom, Kegeles and Heckly (1947*a*) and Watson, Cromartie, Bloom, Heckly, McGhee and Weissman (1947*b*) were concerned solely with the extracellular products of the organism and were interested in their immunising and tissue-damaging properties rather than their aggressive activity. Two materials were recognised in oedema fluid from skin lesions of infected rabbits, a protective antigen and an "inflammatory factor". The "inflammatory factor" was removed from the protective antigen by co-precipitation with calcium phosphate. It contained 60 per cent of polyglutamic acid, produced an inflammatory lesion when injected into the skin, but did not appear to be a protective antigen.

In the present study of the compounds responsible for the virulence of *B. anthracis*, both the intracellular material and the extracellular products of the organism growing *in vivo* (Smith, Keppie and Stanley, 1953*a*, *b*) have been examined. The crude materials were active in tests for aggressins but only the extracellular products were able to immunise animals actively against infection with *B. anthracis* (Keppie, Smith and Harris-Smith, 1953). This paper and a succeeding one are concerned with the fractionation of the extracellular products of the organism. Fractionation of the cellular substance is to be described in a third paper.

Two preparations of the extracellular products have been used. Before it was known that an anthrax toxin was present in the plasma of guinea-pigs dying of anthrax (Smith, Keppie and Stanley, 1955), a mixture of the thoracic and peritoneal exudates of such guinea-pigs, with 10–15 per cent of plasma (called plasma/exudate, Smith *et al.*, 1953*a*), was extracted for compounds responsible for its aggressive and immunising activity. This paper describes how one purified fraction had both activities. More recently, the toxic plasma undiluted with exudate has been examined. The next paper in this series will describe how the use of one of the methods which had been successful in the fractionation of the exudate led to the

recognition of two components of the anthrax toxin, one of which is closely connected with the immunising aggressin.

MATERIALS AND METHODS

Plasma/exudate.—This was collected from guinea-pigs dying of anthrax as described by Smith *et al.* (1953*a*).

Analytical Methods

Total nitrogen.—By Kjeldahl method.

Phosphorus.—By method of Fiske and SubbaRow (1925).

Hexosamine.—By the method of Elson and Morgan (1933) with the modification of Immers and Vasseur (1950).

Ultraviolet absorption spectra.—Observed in the Unicam spectrophotometer.

Amino acids.—Detected by paper chromatography using phenol and collidine as solvents.

Total glutamic acid.—By the method of Housewright and Thorne (1950) after hydrolysis in 3 N-HCl for 16 hr. at 100°. Total protein calculated as glutamic acid was obtained by comparing all the hydrolysate (before paper chromatography) with a glutamic acid standard using the ninhydrin method described by these authors.

Polyglutamic acid.—By the method to be described later by Smith, Zwartouw and Harris-Smith.

Total carbohydrate.—By the Molisch method (Kabat and Mayer, 1948) and the orcinol method of Sørensen and Haugaard (1933).

Citrate.—By the method of Hargreaves, Abrahams and Vickery (1951).

Lipid.—A 1 per cent solution of the material was poured into 10 parts of a mixture of ethanol (50 ml.), ether (50 ml.) and concentrated HCl (3 ml.) at − 20°. After standing overnight a precipitate was removed by centrifugation and the supernatant evaporated to dryness at room temperature. The dry residue was extracted with ether and the ether extract was filtered, evaporated to dryness and weighed.

Assay for Immunising Activity

Serial dilutions of the material were examined in an active immunity test with guinea-pigs which has been described (Keppie *et al.*, 1953). The concentration of the test material was determined which protected 50 per cent of the animals against a subsequent infection with 1000 LD of *B. anthracis* (strain N.P.) spores. This concentration with its fiducial limits ($P = 0{\cdot}95$) in brackets is quoted in the Tables below. The slope of the survival/concentration regression line was shallow for these tests in guinea-pigs and hence wide fiducial limits were obtained when calculations were made on survival rates from experiments on a single fraction. The figures given in the Tables below were obtained from the combined results of a number of similar experiments.

Assays for Aggressive Activity

All fractions examined were clarified before use by centrifugation at 10,000 rev. min. for 15 min. because insoluble precipitates seriously interfered with the assays.

Antiphagocytic activity

Keppie *et al.* (1953) showed that the action of aggressins on the phagocytosis of *B. anthracis* (spores incubated in tryptic meat broth for 20 min. at 37°) by the polymorphs of normal guinea-pig blood could be measured in two ways. The organisms inside the phagocytes of control and experimental samples could be counted on stained films or the organisms not phagocytosed could be counted by a viable count; there was no significant extracellular bactericidal action. The second of these methods was more convenient for routine operation and has been used in this work. Serial dilutions of the various test materials were examined. In the control samples, the phagocytic action of the guinea-pig blood reduced the viable count to 10–40 per cent of the original count. Addition of aggressins increased the survival rate. The activity of a sample is expressed as the lowest concentration in the test mixture which resulted in a percentage survival 10–15 higher than that in the controls. Statistical analysis

showed that this difference had a significance level of 95 per cent or more in 90 per cent of the tests ; the significance level was never lower than 91·5 per cent. Samples from one fractionation were usually compared in the same test since the phagocytic activity of blood samples varied considerably. The assay could detect 2–3-fold differences in activity. The figures given in the Tables below were obtained by averaging 3–8 similar results.

Virulence-enhancing activity

An attenuated strain of *B. anthracis* (Pasteur II) was used. In the absence of added aggressin, intradermal injection (0·2 ml.) of 1×10^8, 1×10^6 and 1×10^5 *B. anthracis* spores killed 22/24, 4/20, 0/24 guinea-pigs (350 g.) respectively. The addition of active material enhanced the killing power of the lower doses. Serial dilutions of test samples were examined and activities are given as the lowest concentration of the material which produced at least 75 per cent and 25 per cent of deaths in groups of 4 guinea-pigs when injected with 2×10^5 and 2×10^4 spores respectively.

Anti-complementary activity.

This was determined by an assay to be described later.

EXPERIMENTAL

Preliminary Fractionation of Plasma/Exudate

A few experiments were based on those of Watson *et al.* (1947*a*). The results confirmed the American work as far as the association of the immunising antigen with β and γ globulins was concerned, but the calcium phosphate method did not, in our hands, separate the protective antigen from polyglutamic acid. These methods of fractionation were discarded in favour of those described below and summarised in Fig. 1.

Barium acetate and ethanol fractionation

Fresh plasma/exudate (1 l., approx. 2·5 per cent w/v non dialysable matter) was concentrated 2–3-fold on collodion ultrafilters at 0–3° and then dialysed for 5 days at 0° and pH 7·5 against distilled water. When freeze-dried the combined ultrafiltrate and diffusate had no immunising activity at 12 per cent ; its antiphagocytic activity was entirely due to sodium citrate content (Allgower, 1947). Isotonic solutions of sodium chloride containing an amount of citrate equal to that present in the test solutions had similar antiphagocytic activity.

Barium acetate (10 g.) was added to the dialysate (500 ml.; immunising activity at 0·14 (0·09–0·20) per cent, antiphagocytic activity at 0·5 per cent, virulence enhancing activity at 2 per cent) and the pH adjusted to 7·0 (± 0·1). The mixture was cooled until ice crystals began to form and then ethanol at − 10° was added slowly with stirring to give a final concentration of 5 per cent v/v. After 4 hr. the precipitate (Fraction I) was removed by centrifugation at − 1° and washed twice with a mixture (100 ml.) of 2 per cent barium acetate solution with 6 per cent v/v of ethanol. The supernatant was cooled and ethanol at − 10° added slowly to give a final concentration of 12 per cent v/v ; the temperature of the mixture was not allowed to rise above − 1°. After standing overnight the precipitate (Fraction II) was collected by centrifugation at − 1° and washed twice with a mixture (100 ml.) of 2 per cent barium acetate solution with 14 per cent v/v ethanol. A further precipitate (Fraction III) was removed at 20 per cent v/v ethanol and the supernatant constituted Fraction IV.

Table I summarises the results of biological and chemical assays on these fractions. After removal of Ba^{++} from the fractions as described below they were dialysed against distilled water and freeze-dried for determination of yields and for chemical analysis. The solids thus obtained would not dissolve completely in water or saline ; hence for use in biological tests the Ba^{++} free solutions were not freeze-dried but dialysed against saline. Fractions I and II contained the active material, the bulk of the plasma protein being in Fraction IV. Polyglutamic acid was concentrated in Fraction I and Fraction II had the greater biological activity. Biological activity did not seem to be connected either with large amounts of carbohydrate or with nucleic acid residues, but Fractions I and II contained large amounts of lipid. The latter explained the insolubility of the materials after freeze-drying. Fraction III contained some active material which could be recovered by re-fractionation.

TABLE I.—*Fractionation of Dialysed Plasma/Exudate by Barium Acetate and Ethanol*

Fraction.*	Yield (per cent).	Anti-phagocytic activity† (lowest active conc. per cent).	Immunising activity† (conc. per cent giving 50 per cent protection).	Glutamic acid (per cent).	Total protein (as glutamic acid per cent).	Carbohydrate (as glucose per cent).	N per cent.	Ash (sulphated per cent).	Lipid content	
									Yield.	Cholesterol.
I	2	0·15	0·054 (0·033–0·089)	38	62	0·7	8·8	16·6	12	+
II	3	0·07	0·022 (0·015–0·032)	17	75	0·9	12·3	4·8	14	+
III	6	0·25	0·14 (0·086–0·24)	11	86	2·5	13·3	—	2	+
IV	65	Inactive at 1·3	5·3 (2·9–9·5)	12	93	1·2	13·2	6·0	1	−

The figures in the Table are the average of those from 4 similar experiments.
* For details of the fractions see text.
† For details of the assays see text.
All fractions contained less than 0·2 per cent P. Ultraviolet light absorption curves of solutions of Fractions I and II showed no maxima at 260 mμ.

Removal of plasma lipid

Removal of the large amount of plasma lipid in the active Fractions I and II was essential to stop spontaneous precipitation from solution which interfered with biological testing and further fractionation. Plasma lipid was successfully extracted from the dehydrated barium precipitates by the following method.

The barium precipitates—Fractions I and II—were washed successively with 50 ml. quantities of 20 per cent v/v ethanol at − 5°, 50 per cent v/v ethanol at − 10°, 70 per cent v/v ethanol at − 20° and two quantities of absolute alcohol at − 20°. They were then shaken with ether containing 5 per cent v/v ethanol (100 ml. for Fraction I and 200 ml. for Fraction II) at − 20° overnight. Finally the precipitates were washed successively with the same series of alcohols as that described above but in reverse order.

The yield of lipid extracted from the barium precipitates was obtained by evaporating, drying, and weighing the alcoholic ether extracts and the absolute

alcohol washings. Fraction I yielded 10 per cent of lipid (20 per cent cholesterol) and Fraction II 11 per cent (25 per cent cholesterol).

Ba^{++} was then removed from the precipitates by the method of Zwartouw and Smith (1956). Fractions I and II in water (60 ml.) at pH 7·5 were shaken overnight with the Na form of Zeocarb 215 (Fraction I 30 g.; Fraction II 15 g.; moist granules). The resin particles were removed from the Ba^{++} free solution, and washed twice with water (15 ml.) at pH 7·5.

This mild method of extraction removed the bulk of the lipids and all the cholesterol without destroying the biological activities of the fractions. After lipid removal, Fraction I and II had antiphagocytic activity and immunising activity at 0·12 and 0·07 per cent, and 0·052 (0·027–0·098) and 0·031 (0·019–0·047) per cent respectively. They still contained a small amount of non-cholesterol lipid (Fraction I, 1·7 per cent ; Fraction II, 2·7 per cent).

Precipitation by 0·5 per cent NaCl and 15 per cent v/v ethanol

A combination of 0·5 per cent NaCl and 15 per cent v/v ethanol precipitated an immunising aggressin from polyglutamic acid in Fraction I, and from a second non-immunising aggressin in Fraction II.

After removal of lipid and Ba^{++} from Fractions I and II the slightly turbid solutions were mixed with NaCl (0·5 per cent), adjusted to pH 7, cooled until ice crystals began to form when ethanol at — 10° was added slowly to give a final concentration of 15 per cent v/v. The mixture was maintained at — 2° overnight and centrifuged at this temperature. The supernatants were dialysed against distilled water at 0°, and freeze-dried. The precipitates were kept separate, dissolved in water (30 ml.) at pH 7, centrifuged from some insoluble matter, dialysed, and freeze-dried. Table II records the yields and biological activities of these fractions.

TABLE II.—*Sub-fractionation of Fractions I and II by 0·5 per cent NaCl and 15 per cent Ethanol at pH 7·0*

	Fraction I.			Fraction II.		
Sub-fraction.	Yield (per cent).	Immunising activity† (conc. per cent giving 50 per cent protection).	Anti-phagocytic† activity (lowest active conc. per cent).	Yield (per cent).	Immunising activity† (conc. per cent giving 50 per cent protection).	Anti-phagocytic† activity (lowest active conc. per cent).
Insoluble in water (pH 7)	9	0·045* (0·019–0·11)	—	7	0·047* (0·02–0·10)	—
Ppt. by NaCl (0·5 per cent) and ethanol (15 per cent v/v)	12	0·015 (0·007–0·033)	0·06	42	0·025 (0·015–0·041)	0·1
Soluble	75	0·81 (0·38–1·72)	0·2	46	0·21 (0·11–0·43)	0·04

* After extraction of the combined materials with phosphate buffer (0·2 μ, pH 8) the residue was inactive at 0·2 per cent.

† For details of assays see text.

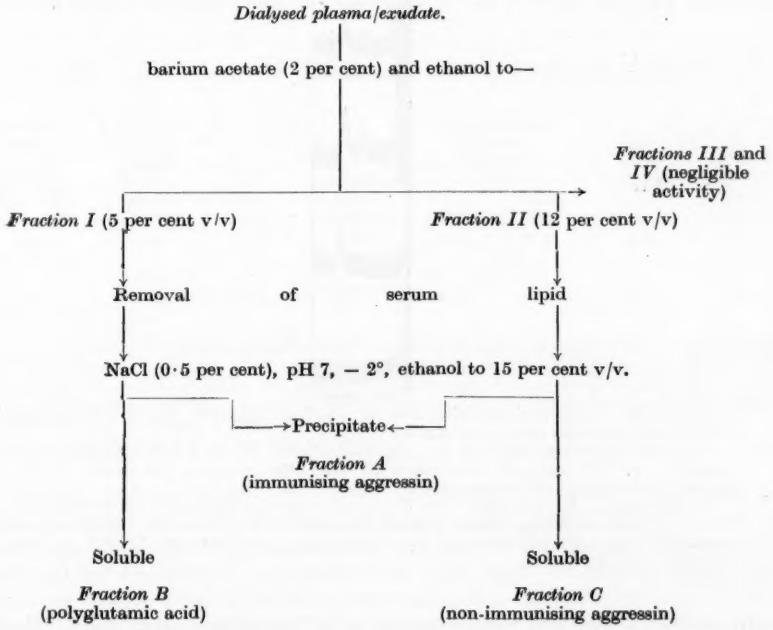
Three main fractions were obtained from the preliminary fractionation of plasma/exudate (see Fig. 1).

Fraction A. The immunising aggressin fraction precipitated by 15 per cent v/v ethanol and 0·5 per cent NaCl from Fractions I and II. Yield from 1 l. plasma/exudate approx. 0·5 g.

Fraction B. The non-immunising material from Fraction I which is soluble in 0·5 per cent NaCl and 15 per cent v/v ethanol. Analysis showed it to contain approx. 70 per cent of sodium polyglutamate. Yield from 1 l. of plasma/exudate approx. 0·25 g.

Fraction C. The material from Fraction II which was soluble in 0·5 per cent NaCl solution containing 15 per cent v/v ethanol. It contained only a small quantity of immunising antigen, but had a high antiphagocytic activity. Yield from 1 l. of plasma/exudate approx. 0·3 g. Fraction C had a high anti-complementary activity being active at 0·05 per cent ; Fractions A and B had no significant anti-complementary activity at 0·4 per cent.

FIG. 1.—Diagram of the preliminary fractionations of plasma/exudate.



Purification of an Immunising Aggressin by Ultracentrifugation of Fraction A

When Fraction A was ultracentrifuged in phosphate buffer (0·2 μ pH 8) at 240,000 × g. in the analytical ultracentrifuge it produced a sedimentation pattern shown in Fig. 2(*a*). Apart from the main component and a small trailing fraction, there was approx. 10–15 per cent of a fast moving component.

Separation of the fast moving component

Fraction A (1·5 g.) in phosphate buffer (0·2μ 100 ml.) at pH 8 was ultracentrifuged in the preparative Spinco ultracentrifuge at 100,000 × *g* (40,000 rev. min.) for 6 hr. at 0–5°.

The supernatant was dialysed against distilled water at 0° and freeze-dried (yield 75 per cent). Its ultracentrifugal diagram is shown in Fig. 2(*b*). The fast

FIG. 2.—Spinco ultracentrifuge diagrams of fractions obtained during the purification of immunising aggressin from Fraction A.

Fractions were dissolved in 0·2 μ phosphate buffer at pH 8 and examined in a field of 240,000 × *g*. Photographs were taken 16 min. after reaching full speed and have been aligned according to the meniscus of the liquid in the cell.

(*a*) Fraction A, 1·7 per cent. (*b*) Ultracentrifugal supernatant from Fraction A, 1·6 per cent. (*c*) Ultracentrifugal deposit from Fraction A, 1·0 per cent. (*d*) Rewashed ultracentrifugal deposit from Fraction A, 1·2 per cent. (*e*) Fraction Y, 0·7 per cent. (*f*) Fraction X, 1·0 per cent.

moving component had been removed as had the biological activity. This fraction had an immunising activity at 0·59 (0·31–1·1) per cent and a slight antiphagocytic activity at 0·25 per cent. It was discarded.

The deposit was re-suspended in the original volume of phosphate buffer and ultracentrifuged at 100,000 × *g* for 4 hr. After repeating this process twice the analytical ultracentrifuge diagram of the deposit was that shown in Fig. 2(*c*). The fast moving component consisted of two parts, the faster moving one being named X and the slower one Y. A small amount of the original main component

remained and was removed by a final 4 hr. ultracentrifugation, see Fig. 2(*d*). The solution of the deposit was dialysed against distilled water at 0° and pH 7·5 and freeze-dried (yield 8 per cent). It had an immunising activity at 0·007 (0·003–0·013) per cent and an antiphagocytic activity at 0·02 per cent.

Separation of components X and Y

The active material (0·5 g.) in phosphate buffer (50 ml.) at pH 8 was ultracentrifuged in the Spinco ultracentrifuge at 100,000 × g for 2 hr. at 0°. The supernatant was ultracentrifuged 3 times for 2 hr. the three deposits being reserved. The final supernatant (component Y) was concentrated on a collodion ultrafilter at 0°, dialysed against ice-cold distilled water at pH 7·5 and freeze-dried. In the analytical ultracentrifuge it produced a diagram shown in Fig. 2(*e*). The deposit was dissolved in the original volume of fresh phosphate buffer and ultracentrifuged for 1½ hr. This was repeated twice. The final deposit (component X) was dialysed and freeze-dried. Its ultracentrifuge diagram is shown in Fig. 2(*f*). The three supernatants were mixed with the three deposits described above, concentrated on a collodion ultrafilter and used again in this part of the process as initial material.

TABLE III.—*Analysis and Biological Activities of Fractions X and Y*

Fraction.	Immunising activity* (conc. per cent giving 50 per cent protection).	Anti-phagocytic activity* (lowest active conc. per cent).	N (per cent).	Lipid† (per cent).	Carbohydrate per cent Orcinol: Glucose.	Carbohydrate per cent Orcinol: Galactose.	Ash (sulphated per cent).	Hexosamine (per cent).
X.	0·012 (0·006–0·028)	0·03	12·0	5·2	4·0	3·7	2·9	2·7
Y.	0·006 (0·003–0·012)	0·02	12·6	4·9	4·2	3·9	2·5	2·6

After hydrolysis with 3N-HCl for 16 hr. at 100° Fractions X and Y showed cysteic acid, aspartic acid, serine, glutamic acid, glycine, threonine, alanine, tyrosine, leucine and isoleucine, valine and methionine, histidine, proline, arginine and lysine on two dimensional paper chromatograms.

* For details of assays see text. Both fractions contained less than 0·2 per cent P and did not strongly absorb light of wavelength 260 mμ.

† The lipid contained less than 2 per cent cholesterol.

The analysis and biological activities of components X and Y are given in Table III. Solutions of both components in phosphate buffer (0·2 μ) at pH 8 showed only one peak, which migrated slowly towards the anode when examined in the micro-cell of the Spinco electrophoresis apparatus. Ultracentrifuge and electrophoresis studies at acid pH were impossible due to the insolubility of the material. Evidence that component X was formed at one stage in the aggregation of component Y which occurs during repeated ultracentrifugation is as follows. There was no evidence of appreciable amounts of component X in the original Fraction A before ultracentrifugation (see Fig. 2(*a*)). When samples containing component Y but no component X were deposited 3 times by ultracentrifugation for 6hr. at 100,000 × *g*, appreciable quantities of component X were present in the product. In the separation of components X and Y a smaller yield of Y (20–30 per cent) and a larger yield of X (60–70 per cent) was obtained than those expected

from a consideration of Fig. 2 (*c* and *d*). A comparison of Fig. 2(*d*) and (*e*), shows that in Fig. 2(*e*) the bulk of component X became so aggregated that it migrated to the bottom of the cell very soon after the ultracentrifuge had reached top speed.

Properties of component Y

Component Y is a purified preparation of an immunising aggressin produced by *B. anthracis in vivo*. Although having a high antiphagocytic activity *in vitro*, it had no virulence-enhancing activity at 0·5 per cent. The analysis shown in Table III indicates that it is a lipoprotein containing a small amount of carbohydrate. The action of trypsin (0·05 per cent w/v) in phosphate buffer at pH 8 for 5 hr. at 37° did not significantly affect the immunising activity of Fraction Y or its antiphagocytic activity. After the removal of lipid from Fraction Y by cold acid-alcoholic-ether the precipitate formed in this solvent was inactive as an immunising antigen at 0·2 per cent but was not tested for antiphagocytic activity because it was largely insoluble in saline. A 0·5 per cent w/v solution of Fraction Y in saline was not toxic to mice when injected intravenously (0·5 ml.) and 0·2 ml. produced no skin reaction when injected intradermally into guinea-pigs. Two guinea-pigs were hyperimmunised by nine intramuscular injections (1 ml.) of Fraction Y (0·5 per cent w/v) at one week intervals. The serum of these guinea-pigs specifically neutralised the toxic action of the anthrax toxin in a mouse lethality test and in a skin oedema test (*cf.* the behaviour of hyperimmune horse serum prepared against the antigen of *B. anthracis* produced *in vitro*—Smith *et al.*, 1955). Fraction Y (1 ml., 0·5 per cent w/v) did not protect guinea-pigs when injected 8–12 hr. before challenge with 1000 LD of *B. anthracis* spores.

Sodium Polyglutamate from Fraction B

The purification of sodium polyglutamate from Fraction B, criteria of purity, and its biological and chemical properties are described by Zwartouw and Smith (1956). The purified sodium polyglutamate contained less than 0·2 per cent of foreign amino acids and was active at 0·8 per cent in the antiphagocytic test. It was active at 4–2 per cent in the virulence-enhancing test but it was inactive at 2 per cent as an immunising antigen.

Fraction C

This material was treated as follows. Ethanol was added to a 2 per cent solution in 0·5 per cent aqueous NaCl to a final concentration of 15 per cent v/v. A small precipitate which formed at — 2° was removed by centrifugation. The supernatant was dialysed and freeze-dried. A precipitate of polyglutamic acid was removed after adding ethanol (5 per cent v/v) to a 2 per cent w/v solution of the freeze-dried solid in 2 per cent aqueous barium acetate at pH 7. The material containing antiphagocytic and anti-complementary activity was then precipitated from the supernatant by adding ethanol to a final concentration of 12 per cent v/v. After removal of Ba^{++} by an ion exchange resin as described above and dialysing against 0·2μ phosphate buffer at pH 8, a small amount of immunising antigen was deposited from the solution by ultracentrifugation at 100,000 × *g* for 6 hr. The ultracentrifugal supernatant was dialysed against distilled water at 0° and freeze-dried. Yield from Fraction C 20 per cent. It had N 13·2, sulphated ash 2, P less than

0·2, and carbohydrate (Molisch as glucose) 1 per cent. It was antiphagocytic and anti-complementary at 0·03 per cent but had no immunising activity at 0·2 per cent.

Biological Tests on Fractions from Normal Guinea-pig Plasma

The immunising aggressin present in Fraction A was obviously a specific product of *B. anthracis* and polyglutamic acid in Fraction B was also derived from the pathogen. It was not certain however that the anti-complementary but non-immunising aggressin in Fraction C was a specific product of the organism. To shed some light on this question appropriate fractions of normal guinea-pig plasma were examined. The plasma was fractionated as described above for plasma/exudate, Fraction II being split into Fractions A and C after removing the serum lipid. No complications arose during this fractionation.

Fraction C from normal guinea-pig plasma had neither antiphagocytic nor anti-complementary activity at 0·2 per cent. The anti-complementary, non-immunising aggressin from plasma/exudate is therefore formed as a result of the growth of *B. anthracis* in the host. Protein fractions from the intracellular material of *B. anthracis* (Smith, Zwartouw and Harris-Smith—to be published) are anti-complementary and antiphagocytic but not immunising antigens. The material from plasma/exudate resembles these protein fractions in being precipitated by barium acetate and 12 per cent v/v ethanol but not by 0·5 per cent NaCl and 15 per cent v/v ethanol. A precipitate formed when a solution of the material from plasma/exudate was mixed with the serum of guinea-pigs hyperimmunised with a mixture of the proteins from the bacteria. The material isolated from plasma/exudate may therefore be bacterial protein set free from the surface or by lysis of the organism.

Fraction A from normal serum which was neither antiphagocytic nor anti-complementary at 0·2 per cent did not contain a small fast moving ultracentrifuge component.

DISCUSSION

It seems that the speculation made by Bail (1904) that the immunising and aggressive activities of exudates from animals infected with *B. anthracis* are due to the same substance may be substantially correct. Fraction Y isolated by methods described above from infected guinea-pig exudates is a powerful immunising antigen and has marked antiphagocytic properties.

This preparation from anthrax exudates is a lipoprotein containing a small carbohydrate moiety and it is different from the immunising preparation produced *in vitro* (Wright, Hedberg and Slein, 1954; Belton and Strange, 1954) and purified by Strange and Belton (1954) although the two materials had similar immunising activity in guinea-pigs and antiphagocytic activity *in vitro* (see Table IV). Their behaviour in the ultracentrifuge is quite different and this is important in connection with work described in the next paper. The antigen prepared *in vitro* sediments slowly, whereas Fraction Y rapidly deposits. An approximate value for the sedimentation constant of Fraction Y is 15, which is not unlike that of the small S20 component of normal plasma (Pedersen, 1945; Oncley, Scatchard and Brown, 1947). It is possible that the immunising agressin is in some way connected with this S20 component, *e.g.*, a small molecular component from the organism may have combined with it.

TABLE IV.—*Immunising Aggressins of* B. anthracis *Produced* in vivo *and* in vitro.

Preparation.	N (per cent).	Carbohydrate residues (per cent).	Lipid (per cent).	Approx. sedimentation constant.	Anti-phagocytic activity* (lowest active conc. per cent).	Immunising activity (conc. per cent giving 50 per cent protection).
In vitro (Strange and Belton, 1954)	14·2	Less than 1	—	3	0·03	0·007 (0·002–0·024)
In vivo (Fraction Y)	12·6	4–7	5	15	0·02	0·009 (0·002–0·035)

* For details of the assays see text. Both preparations were not toxic and had no virulent enhancing activity, but they produced in suitable animals antisera which neutralised the toxic and oedema-producing activities of anthrax toxin.

It is probable that the immunising aggressin isolated as Fraction Y is a converted form of the biologically active material present in the original plasma/exudate. This exudate had a slight tissue-damaging activity (Keppie *et al.*, 1953) and a marked virulence-enhancing activity apart from its immunising and antiphagocytic properties. During the process described above both the tissue-damaging activity and the virulence-enhancing activity were destroyed and were absent from Fraction Y. There is a close connection between the tissue-damaging anthrax toxin and the non-toxic immunising antigens (Smith *et al.*, 1955). Furthermore the adverse effects of the fractionation procedures described here on the tissue-damaging properties of plasma containing this toxin were soon recognised (Smith *et al.*, 1955). It will be seen in a succeeding paper that Factor I of the toxin probably becomes converted to Fraction Y during the fractionation process.

Polyglutamic acid, obtained in a homogeneous condition from plasma/exudate, is an aggressin and its properties in this connection are discussed more fully by Zwartouw and Smith (1956). It is not an immunising antigen for guinea-pigs and, as will be described later, is not connected with the tissue-damaging anthrax toxin (*cf.* Watson *et al.*, 1947*b*). This work suggests that the "inflammatory factor" of Watson *et al.* (1947*b*) was a mixture of the polyglutamic acid with a small amount of the anthrax toxin.

The non-immunising, anti-complementary, antiphagocytic protein isolated from Fraction C is produced by the growth of *B. anthracis* in the host. It resembles the bacterial protein isolated from the organism and may have been set free from the surface of the organism or by its lysis.

SUMMARY

A purified fraction has been obtained from the thoracic and peritoneal exudate of guinea-pigs dying of anthrax which is an immunising antigen and has marked antiphagocytic properties. This immunising aggressin, which sediments rapidly in the ultracentrifuge, is a lipoprotein containing a small amount of carbohydrate residues. It is different from a preparation of the immunising antigen of *B. anthracis* produced *in vitro*.

Sodium polyglutamate has been isolated from the infected exudates in a homogeneous condition. It is an aggressin but not an immunising antigen.

A third fraction which, like the bacterial protein, had marked anti-complementary and antiphagocytic properties, has been isolated from the exudates.

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A METHOD FOR ASSAYING ANTHRAX IMMUNISING ANTIGEN AND ANTIBODY

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THE experiments described were planned with a view to developing a skin test in rabbits as a convenient and reasonably accurate assay technique for immunity studies with anthrax.

An important limiting factor in studies on the production of anthrax antigen (Wright, Hedberg and Slein, 1954 ; Belton and Strange, 1954) and in its purification (Strange and Belton, 1954) has been the absence of a simple, reliable and reasonably rapid method of assay. A long and not very sensitive active immunity test in rabbits or guinea-pigs was the only method available. Similarly the titration of antibody was limited to a passive protection test in the rabbit, which was far from being accurate or sensitive. Complement fixation or precipitation tests were shown to be unreliable, at least in studies with crude antigen preparations.

The demonstration by Smith, Keppie and Stanley (1955) of a toxin produced by *Bacillus anthracis in vivo* offered possibilities for the development of a suitable method of assay. Not only was the toxin lethal but it produced characteristic skin lesions in guinea-pigs and rabbits ; further, it was specifically neutralised by anthrax antibody produced either by immunisation of animals with an avirulent spore vaccine or with the soluble antigen produced *in vitro*. This latter substance is not toxic and produces no trace of reaction following intradermal injection.

Smith *et al.* (1955) observed that the skin lesion produced in the guinea-pig by intradermal injection of the toxin developed over a period of 24 hr. At maximum size it consisted of an oedematous weal with evidence also of vascular damage in the form of haemorrhage in the deeper vascular areas of the corium. The skin surface remained intact and within 7 days all trace of reaction had disappeared. When the toxin was neutralised with antitoxin *in vitro* and then similarly injected no trace of skin reaction was detectable 24 hr. later. We have found that in rabbit skin the same well defined oedematous weal is produced but without the erythema and it is at maximum size about 18 hr. after intradermal injection. Further, the sensitivity of the rabbit skin is about five to six times greater than that of the guinea-pig. In selecting the rabbit for the purpose of the present experiments we took into account not only its higher sensitivity but also the practical point of greater available surface area per animal.

METHODS

Test toxin.—This was kindly prepared for us in the freeze-dried state by Dr. Keppie, using the method previously described (Smith *et al.*, 1955). It was stored at 0–4° in small screw-cap bottles each containing originally 3 ml. of the toxic plasma. Throughout the work two large batches of freeze-dried toxin were prepared. From each three bottles were taken by

random selection, reconstituted in physiological saline, pooled and titrated intracutaneously in the rabbit. A dose of 0·2 ml. was injected which contained 0·1 ml. of the toxin dilution and 0·1 ml. of normal horse serum. The least amount of toxin producing lesions with a mean diameter of 15–18 mm. was selected as the "test dose." Thereafter, for each experiment a single bottle was taken, the toxin reconstituted and used immediately at the test dose level. There was no evidence of deterioration of the toxin in the dried state over many months. Minor variations from test to test were noted in the size of the lesions produced; by a suitable system of controls their effects were eliminated.

Laboratory standard anti-serum.—This was prepared in the horse by repeated injection of alum-precipitated antigen (Belton and Strange, 1954). It protected rabbits when given intravenously in 3 ml. amounts 24 hr. prior to intracutaneous injection of anthrax spores containing about 250 A.L.D. It was stored at 0–4° in liquid form without antiseptic for over two years and showed no evidence of deterioration. This certainly was borne out over the period covered by the present experiments.

It was found that 0·1 ml. of a dilution of 1/300 or less of the serum added to the test dose of toxin also contained in 0·1 ml. completely prevented the skin reaction. At a serum dilution of 1/400 there was nearly complete inhibition of toxin action. Frequently the reaction obtained was not measurable and was recorded as "slight" or "trace." Thus, for example, using serum at a starting dilution of 1/400, of 125 test animals selected at random from the full series of over 600 rabbits used, the records show 18 animals with measurable lesions, 63 with no skin lesion and 44 with slight or trace lesions; all were randomly distributed. When the anti-serum was used at a dilution of 1/800 the lesions produced by the test toxin were indistinguishable from control lesions produced by the test dose of toxin in the presence of an equal amount of homologous normal serum.

Animals.—Albino rabbits (Copenhagen strain) weighing 2 kg. or more were used. The thin skin found on the lighter, younger rabbits, and also on the abdomen of those used, was unsuited for test purposes. A strip of fur approximately 3 in. (7·5 cm.) wide was shaved from each side of the back. Two rows of five injections were made on each side in the thoracic and lumbar areas. An occasional animal (18 out of 625, or 3 per cent) has failed completely to respond to the test dose of toxin.

Preparation of materials for injection.—For the titration of antibody the toxin was first diluted so that the test dose was contained in each 0·1 ml. of solution. To aliquots of this preparation equal volumes of suitable dilutions of anti-serum were added, mixed and stored for 2 hr. at 0–4° before the intradermal injection of 0·2 ml. amounts. Normal serum controls were similarly prepared and injected.

In titrating antigen the standard anti-serum was diluted 1/100. To portions of this equal quantities of appropriate dilutions of antigen were first added. The mixtures were incubated in a water bath for 2 hr. at 37°, then stored at 0–4° overnight. After centrifuging to remove small amounts of precipitate, an equal volume of toxin carrying a test dose in 0·1 ml. was added and mixed. After 2 hr. at 0–4° intradermal injections of 0·2 ml. amounts were made in rabbit skin. The dose of antibody thus given (≡ 0·1 ml. 1/200 dilution) was twice the amount previously found to be marginal in neutralising a test dose of toxin (see above). Standard antibody plus toxin controls were similarly prepared, the antigen solution being replaced by saline. When antigen capable of combining with the antibody was present in the mixture in sufficient amount then a skin lesion would be expected to develop. By a process of dilution of the antigen a point would be reached where its effect would be masked by excess antibody.

Procedure for the inoculation of rabbits and recording of results.—There was some variation between animals in their response to the toxin. This difficulty was overcome by using at least four rabbits in any titration and by including a full series of control inoculations on each animal. This included the test dose of toxin + standard antitoxin at dilutions of 1/200 and 1/400: similarly the toxin + normal serum in the same dilutions as those used for test material and the test dose of toxin in saline. To overcome any variation in degree of reaction according to the site of inoculation the Latin Square principle of randomisation was adopted and a different scatter used for each animal.

The results were recorded 18–20 hr. after inoculation by measuring the lesions across two diameters at right angles, and the mean diameter taken. As noted above, the end-point of titration not infrequently gave a lesion that could be detected by palpation, but could not be measured with any degree of accuracy. Such lesions were recorded as "slight" or "trace" results.

RESULTS

The sensitivity of the test is of an entirely different order from, for example, that of the titration of diphtheria antitoxin in the guinea-pig skin. Differences less than that shown by twofold dilutions cannot be detected with any degree of accuracy. The reason for this is to be found partly in the fact that only three types of skin reaction can be assessed—the fully developed and measurable lesion, the slight or trace reaction noted above, and the completely negative response. The results are therefore not suitable for statistical analysis. This is not important if one accepts a twofold difference in potency as the maximum degree of differentiation to be expected, for a simple inspection of results clearly shows the relative potency of an antigen or an antibody in comparison with laboratory standards. We proved this to our satisfaction by first titrating a series of antigens and antibodies. The experiments were repeated after the antigens and antibodies had been randomised by an independent worker in a manner unknown to us. The results were in perfect agreement with the original observations. Examples of the test in operation follow.

Table I shows the results obtained in titrating purified *in vitro*-grown antigen (Strange and Belton, 1954). According to our method of recording, the results obtained by the use of 25 μg. of antigen per ml. show the end-point of titration; twice this amount neutralises all antibody and allows the development of lesions closely similar to those given by the toxin control. On the other hand, 12·5 μg. fails to show any evidence of serum neutralisation. By coincidence, 25 μg. of this particular antigen is the lowest dose that produces full active protection in rabbits.

TABLE I.—*Titration of Purified Antigen*

Antigen μg./ml.	Standard antibody/ml.	Rabbit. 1.	2.	3.	4.	5.	6.	7.	8.	9.	10.
100	0·01	17*	15·5	17·5	18·5	18·5	17	15	17	15·5	18
50	,,	14·5	16	16·5	17·5	19·5	19	Sl	Sl	17·5	19·5
25	,,	Sl	0	0	16·5	Sl	Sl	Sl	Tr	15	Tr
12·5	,,	0	0	0	0	0	0	0	0	0	0
Nil	,,	0	0	0	0	0	0	0	0	0	0
,,	0·005	Tr	Tr	Sl	0	Tr	Sl	Sl	Sl	0	Sl
Toxin control		16	20	19	14	17	15	18·5	20	13·5	15·5

* Mean diameter of lesion in mm. Sl = slight reaction; Tr = trace reaction.

In Table II the results of two experiments are shown, in which our laboratory standard antigen was titrated. This material is held at 0° as a freeze-dried culture filtrate to which 0·5 per cent normal serum had been added as a stabiliser during the drying process. In both experiments the end-point of titration is clearly shown at the 3·25 mg. level of antigen. Combined data from 8 active immunisation experiments in rabbits with this material showed that 3·25 mg. protected 41/48 and 1·08 mg. 29/50 animals; 16 mg. protected 50/50 rabbits.

The use of the test in titrating the relative potencies of immune serum is demonstrated in Table III. As reported elsewhere (Belton and Strange, 1954; Henderson, Peacock and Belton, 1956) monkeys can be rendered immune to anthrax by two injections of *in vitro*-produced antigen. In a recent experiment designed to test the duration of immunity, a group of monkeys immunised 12

TABLE II.—*Titration of Laboratory Standard Antigen (Freeze-dried Culture Filtrate)*

Antigen mg./ml.	Standard antibody/ ml.	Rabbits. Experiment 1. 1.	2.	3.	4.	5.	Experiment 2. 1.	2.	3.	4.	5.
13	0·01	18*	16	19·5	19·5	16·5	17	18·5	16·5	19	15·5
6·5	„	17·5	12	19·5	16·5	20·5	16	15·5	18	17·5	19·5
3·25	„	15	0	15·5	Tr	0	15	0	Sl	16·5	Sl
1·625	„	0	0	0	0	0	0	0	0	0	0
Nil	„	0	0	0	0	0	0	0	0	0	0
„	0·005	0	Tr	Tr	0	12	0	Sl	0	Sl	0
Toxin control		21·5	20	18	21·5	22	18	20	Sl	17·5	16·5

* Mean diameter of lesion in mm. Sl = slight reaction ; Tr = trace reaction.

months earlier were exposed to a respiratory challenge dose of anthrax spores carrying approximately 16 A.L.D. They all survived and all control animals died of anthrax. One week prior to challenge, and again 4 weeks after, they were bled and serum collected for titration. Table III summarises the results obtained with the serum from one animal which is characteristic of the group. Before challenge the end-point of titration is shown at a dilution of 1/3. After challenge a dramatic rise in antibody is witnessed, with the end-point at a dilution of 1/256. Unfortunately the picture is incomplete, for there is no record of immunity level soon after immunisation. However, this form of test clearly shows that, whereas the animals survived the challenge dose, there must have been a degree of infection leading to a sharp immunity response.

TABLE III.—*Rise in Antibody Level in an Immunised Monkey following a Respiratory Challenge Dose of Anthrax Spores*

Serum dilution. 1:	Before challenge. Rabbit No. 1.	2.	3.	4.	After challenge. Rabbit No. (as opposite). 1.	2.	3.	4.
Undiluted	0	0	0	0	—	—	—	—
3	14·5*	Sl	Sl	14	—	—	—	—
9	14·5	17	17·5	14·5	—	—	—	—
27	17·5	14·5	18	15·5	—	—	—	—
64	—	—	—	—	0	0	0	0
128	—	—	—	—	0	0	Sl	Sl
256	—	—	—	—	15	14·5	Sl	15
512	—	—	—	—	14·5	15·5	14·5	16
Standard antibody 200	0	0	0	0				
„ „ 400	0	0	Tr	Sl				
Normal monkey undil.	15·5	15·5	14	19				
Toxin control	15·5	17·5	16·5	17				

* Mean diameter of lesion in mm. Sl = slight reaction ; Tr = trace reaction.

DISCUSSION

The type of test described will facilitate systematic studies on the metabolism of *B. anthracis* leading to the production of protective antigen *in vitro* as well as research on the purification and identification of the active principle. There is evidence also that it will be valuable in immunity studies. There are, however,

certain limitations. It is not as sensitive a test as might be desired, since titration can show differences no closer than twofold. The variability in the response of rabbits necessitates the use of several animals per test, although in any test 2 to 4 specimens of antigen or antibody may be compared with standard preparations. Finally, the minimum antibody level necessary to ensure protection against either respiratory or parenteral infection with anthrax spores might be such that it cannot be detected by the present method of test. Indications of this have been given by the examination of sera from monkeys proven to be immune. Further, sera from 31 humans who had been given two injections of antigen (sufficient to develop full protection in the monkey) have consistently failed to show evidence of toxin-neutralising activity in the skin test. However, of 89 humans given a third dose of antigen about 12 months after the second injection, 88 responded in such manner that the skin test clearly showed marked neutralising activity of the serum; one individual failed to respond. This may mean, of course, that the present method of immunisation for humans is inadequate. The important point perhaps is that with this new method of test it may be possible to arrive at a more accurate assessment of the immunity level essential for complete protection.

SUMMARY

Sterile plasma from guinea-pigs dead of anthrax produces a characteristic skin lesion in rabbits, which reaction can be completely neutralised by anthrax anti-serum. This has formed the basis of the development of a skin test for the titration of *in vitro*-produced anthrax antigen (which is non-toxic) and of anthrax anti-serum. It is possible to detect twofold differences in potency of either antigen or antibody.

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THE PRODUCTION OF ANTIBODIES IN COTTON RATS BY LIVING AND KILLED POLIOMYELITIS VIRUSES[1]

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FOLLOWING Armstrong's adaptation of the Lansing strain of Type 2 poliomyelitis virus to rodents, Pinto (1948) showed that Type 2 virus produced neutralising antibodies in cotton rats after parenteral injection by peripheral routes. It has now been shown that after similar injections all three types of poliomyelitis viruses, either living or formalinised, are capable of producing antibodies in these animals. The sera produced were used to compare, by the cross neutralisation technique, strains of virus within each serological type. These strains were isolated in Minnesota from 1946 to 1954 (Syverton, 1954). Cotton rats were also used to test the antigenic potency of three poliomyelitis vaccines which had been used for the inoculation of human subjects.

MATERIALS AND METHODS

Cotton rats 6–12 weeks old for the cross-neutralisation, and 7–9 weeks for the comparison of the vaccines, were used in these experiments. They were injected by the intramuscular or intraperitoneal routes.

Virus strains.—Mahoney (Type 1), Yale SK (Type 2) and Saukett (Type 3) served as type strains. Field strains isolated in Minnesota since 1946 were used in the cross-neutralisation tests. They were designated by a method similar to that used for the influenza viruses (Chu, Andrewes and Gledhill, 1950) in which the type of virus is followed by the location, the local laboratory number and the year of isolation. This method gives more information about the strain than the usual ones used with the poliomyelitis viruses. These strains were isolated from the faeces of paralytic and non-paralytic cases and were selected at random. All were isolated in HeLa cells. The Type 1 strains had been passed 4–8 times in these cells, the Type 2 strains from 3–5 times and the Type 3 strain 3–7 times before being injected into cotton rats. The same passage material was used in the neutralisation tests and for rat inoculation. Two strains of Type 1 were used for each year from 1946 to 1953 except for 1947, one of Type 2 from years 1948, 1949, 1951 and 1954 and one of Type 3 for all years except 1946 and 1949. The years omitted were those in which no virus of a given type was isolated (Tobin, Brunner, Ellwood and Syverton, 1954). All the Type 1 strains were capable of infecting cynomolgus monkeys after intramuscular injection as indicated by antibody response, while both of those from 1946, 1948 and 1949, one from years 1950 and 1952 and none from 1951 and 1953 produced paralysis in those animals. Three of the Type 1 strains were found to survive for 4–8 days in the C.N.S. of 4-week-old mice after intraspinal injection as compared to 24 hr. or less with the control Mahoney strain. There was no relation between these effects and the infective titre in HeLa cells. No comparisons of the biological activities of the Type 2 and 3 strains were made in animals.

Virus vaccines.—Three trivalent formalinised poliomyelitis vaccines, designated A, B and C, were tested.

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Antibody titrations.—Serum (1–1·5 ml.) was obtained by cardiac puncture from anaesthetised rats before and after inoculation. After inactivation at 56° for 30 min. antibody was titrated using HeLa cell cultures (Syverton and Scherer, 1954), which were maintained by Hanks' basic salt solution (BSS), containing 5 per cent chicken serum, 0·1 per cent Difco yeast extract (Robertson, Brunner and Syverton, 1955), 0·25 per cent glucose, 0·07 per cent sodium bicarbonate and antibiotics. The dilutions of serum were made in BSS containing 0·008 per cent sodium bicarbonate and an equal volume of virus (0·25 ml.), in the same medium, added so that each culture tube received approximately 50 tissue culture ID_{50} of virus. In most tests serum-virus mixtures were held for 1 hr. at room temperature and then overnight at 4° before being added to the cell culture, since it was found that the titres were often higher and never lower than those found if the overnight stage was omitted. No differences in test virus titres were noted in the two procedures. The tests were read after 3 and 6 days. In the cross-neutralisation tests 4 tubes were used for each 3-fold dilution with Type 1 and for each 4-fold dilution of serum with the other types, and the $T.C.PD_{50}$ end-point was calculated by the method of Reed and Muench. In these experiments serum dilutions were held at − 20° until required. When comparing the antigenic potency of vaccines only one tube per dilution of serum was used, and the highest one protecting against the cytopathic effect of virus was taken as the end-point.

RESULTS

Production of Antibodies

In a few preliminary experiments in which cotton rats were injected with living Mahoney virus it was found that after one injection antibody appeared by the beginning of the second week, rose to a maximum during the third week and then slowly fell to about a quarter of its highest titre by 6–8 weeks. The titres following intramuscular injection tended to be somewhat higher than those following intraperitoneal injection.

TABLE I.—*Relation of Type* 1 *Virus Titre in the Inoculum to Antibody Production in Cotton Rats* (*Minnesota Strains*, 1946–53)

Log of $TCID_{50}$ in inoculum (0·5 ml.).	Number of rats producing antibody /number injected.
$<5·4$	1/4
5·4–6·4	11/14
$>6·4$	10/10

As can be seen from Table I, large amounts of Type 1 virus were needed to produce titres of 10 or more after one intramuscular injection, the response being related to the infective titre of the inoculum rather than to the strain of virus used. When the inoculum contained more than $10^{6·4}$ tissue culture infective particles, all the animals tested produced antibody, when between $10^{5·4}$ and $10^{6·4}$, 11 out of 14 animals, and when below $10^{5·4}$, only 1 out of 4 did so. The titres varied up to 1024, depending on the rat rather than on the inoculated strain.

Similarly, large amounts of Type 3 were necessary for antibody production following a single intramuscular injection. With both Types 1 and 3 a second injection, 4–6 weeks after the first, further increased the antibody titre (Table II).

The titre after one intramuscular injection of the Type 2 strains varied between 16 and 512, values similar to those of Pinto, who used mice for the neutralisation test. No antibody to heterologous types was produced, nor was any animal found with pre-existing antibody to the three virus types.

TABLE II.—*Antibody Response in Cotton Rats after 1 and 2 Injections of Live Poliomyelitis Virus.*

Rat No.	Virus.	Log of titre of inoculum.	Log of antibody titre 14 days after 1st injection.	Log of antibody titre 10 days after 2nd injection.
1	Type 1 (Mahoney)	6·6	1·4	3·1
2			1·9	3·1
3			2·3	3·4
4			1·5	2·3
5	Type 3 (Saukett)	5·4	1·8	2·9
6			<0·35	1·3

Interval between the first and second injection, 4–6 weeks.

Cross-neutralisation Tests

One intramuscular injection of live virus of adequate titre produced a sufficient antibody response for cross-neutralisation tests. Sera obtained against 14 strains of Type 1 virus, 4 of Type 2 and 6 of Type 3 were used to compare, by the cross-neutralisation technique, the immunological relation within each type. The antigenic ratio, as used by Chu *et al.* (1950) for comparison of influenza strains, was determined for each pair of poliomyelitis viruses. This ratio is the square root of the product of the heterologous titres divided by the homologous titre in each case. The nearer the ratio is to unity the more closely related antigenically are the strains. Values greater than unity reflect on the accuracy of the test and should only occur when strains are very similar.

TABLE III.—*Antigenic Ratios of Type 1 Poliomyelitis Viruses (Minnesota 1946–53).*

Virus strain.	1/Minn. 91/53.	1/Minn. 3/52.	1/Minn. 4/51.	1/Minn. 6/50.	1/Minn. 1/49.	1/Minn. 27/48.	1/Minn. 2/46.	Type 2 (Yale-SK).	Type 3 (Saukett).
1/Minn. 91/53	1/1	1·7/1	1·6/1	1/1·2	1·1/1	1·7/1	1/1	<1/450	<1/900
1/Minn. 3/52	1·7/1	1/1	1·5/1	1/1·6	1/1·2	1·7/1	1·4/1	<1/860	<1/1700
1/Minn. 4/51	1·6/1	1·5/1	1/1	1/1·4	1/1	1·7/1	1·5/1	<1/490	<1/1000
1/Minn. 6/50	1/1·2	1/1·6	1/1·4	1/1	1/1·3	1·4/1	1/1·1	<1/1350	<1/2750
1/Minn. 1/49	1·1/1	1/1·2	1/1	1/1·3	1/1	1·7/1	1·2/1	<1/490	<1/1000
1/Minn. 27/48	1·7/1	1·7/1	1·7/1	1·4/1	1·7/1	1/1	1·4/1	<1/450	<1/900
1/Minn. 2/46	1/1	1·4/1	1·5/1	1·1/1	1·2/1	1·4/1	1/1	<1/560	<1/1120
Type 2 (Yale-SK)	<1/450	<1/860	<1/490	<1/1350	<1/490	<1/450	<1/560	1/1	N.T.
Type 3 (Saukett)	<1/890	<1/1700	<1/1000	<1/2750	<1/1000	<1/900	<1/1120	N.T.	1/1

TABLE IV.—*Antigenic Ratios of Type 2 Poliomyelitis Viruses (Minnesota Strains, 1948–1954).*

			Antisera.				
Virus strains.	2/Minn. 1/48.	2/Minn. 1/49.	2/Minn. 1/51.	2/Minn. 1/54.	Yale-SK.	Type 1 (Mahoney).	Type 3 (Saukett).
2/Minn. 1/48	1/1	1·1/1	1·1/1	1·1/1	2·2/1	<1/200	<1/320
2/Minn. 1/49	1·1/1	1/1	1/1·6	1/2·2	1/1	<1/970	<1/1400
2/Minn. 1/51	1/1·1	1/1·6	1/1	1/1·4	1/1·6	<1/350	<1/560
2/Minn. 1/54	1·1/1	1/2·2	1/1·4	1/1	1/1	<1/450	<1/700
Yale-SK	2·2/1	1/1	1/1·6	1/1	1/1	<1/110	<1/180
Type 1 (Mahoney)	<1/200	<1/970	<1/350	<1/450	<1/110	1/1	N.T.
Type 3 (Saukett)	<1/320	<1/1400	<1/560	<1/700	<1/180	N.T.	1/1

TABLE V.—*Antigenic Ratios of Type 3 Poliomyelitis Viruses (Minnesota Strains, 1948–1954).*

Virus strains.	Antisera. 3/Minn. 1/48.	3/Minn. 1/50.	3/Minn. 1/51	3/Minn. 1/52.	3/Minn 1/53.	3/Minn. 1/54.	Type 1 (Mahoney).	Type 2 (Yale-SK).
3/Minn. 1/48	1/1	1/1	1/1	1/1	1/1	1/2	<1/630	<1/1000
3/Minn. 1/50	1/1	1/1	1/1·2	1·2/1	1·2/1	2/1	<1/160	<1/250
3/Minn. 1/51	1/1	1/1·2	1/1	1/1·7	1/1·4	1/1·4	<1/360	<1/570
3/Minn. 1/52	1/1	1·2/1	1/1·7	1/1	1/1	1/1	<1/220	<1/390
3/Minn. 1/53	1/1	1·2/1	1/1·4	1/1	1/1	1·4/1	<1/1300	<1/2000
3/Minn. 1/54	1/2·0	2·0/1	1/1·4	1/1	1·4/1	1/1	<1/430	<1/690
Type 1 (Mahoney)	<1/630	<1/360	<1/360	<1/220	<1/1300	<1/430	1/1	N.T.
Type 2 (Yale-SK)	<1/100	<1/250	<1/570	<1/390	<1/2000	<1/690	N.T.	1/1

The Type 2 and 3 strains were all run against each other, while the Type 1 strains were divided into two groups of 7, those with the higher titres produced by one of the representative strains of each year being tested in one group and those with the low titres in the other. Sera prepared against any two virus strains were tested by cross-neutralisation with similar amounts of each virus. As can be seen from Tables III to V, the antigenic ratios fell around unity with all three types of virus. With Type 1 only the results with the higher titre group are given, but those with the lower ones gave similar results. Although the type strains of 1 and 3 were not used in the cross neutralisation tests, the titres of the sera obtained with them were of the same order as those therein (Table VI).

TABLE VI.—*Reciprocals of Titres of Antibody in Individual Cotton Rats after Graded Doses of Formalinised Poliomyelitis Vaccine.*

Vaccine A. Dose.	Rat.	Antibody titre to Type 1.	Type 2.	Type 3.	Vaccine B. Dose.	Rat.	Antibody titre to Type 1.	Type 2.	Type 3.	Vaccine C. Dose.	Rat.	Antibody titre to Type 1.	Type 2.	Type 3.
2 ml.	1	96	96	128	4 ml.	1	24	96	6	10 ml.	1	96	1536	96
	2	12	32	6		2	24	384	6		2	80	96	96
	3	24	96	24		3	12	24	384		3	7	1536	<7
	4	24	10	<6		4	96	6	96		4	96	1536	96
	5	96	96	<6		5	24	1536	24		5	<6	24	96
											6	96	384	384
											7	24	384	24
0·5 ml.	6	<6	24	<6	0·4 ml.	6	24	24	6	1 ml.	8	122	448	384
	7	24	28	96		7	<6	24	96		9	<6	448	24
	8	384	6	<6		8	96	24	96		10	6	1536	24
	9	24	24	6		9	6	256	24		11	24	448	96
	10	<6	384	6		10	24	24	6		12	11	96	<11
						11	96	—	—		13	6	96	6
											14	<6	24	6
0·125 ml.	11	<6	24	24	0·04 ml.	12	<6	<6	<6	0·1 ml.	15	<6	<6	<6
	12	<8	8	<8		13	<6	24	<6		16	<6	<6	<6
	13	<6	96	<6		14	24	96	6		17	6	96	384
	14	384	96	<6		15	6	6	24		18	<6	384	<6
						16	6	24	<6		19	8	64	<6
											20	6	128	8

Two doses were given to each cotton rat at an interval of 14 days and the animals bled 14 days later.

Comparison of Antigenic Potency of Vaccines

With formalinised vaccines, intraperitoneal and intramuscular injections produced similar antibody levels when the Mahoney strain was used for testing antibody to Type 1. Injections spaced at 2-week intervals were nearly as effective as those given at a month's interval or those following three injections, two within a week followed by a third four weeks later.

In order, therefore, to compare the antigenic potency of the three vaccines, two intraperitoneal injections at an interval of 14 days were used. Three groups of 4–7 rats were each injected with different doses of vaccine and bled 14 days after the second inoculation. Although the regime was similar, the rats injected with vaccines B and C were from a different batch from those inoculated with A, but the ages and the source of the animals were the same. All rat sera were tested with each virus in the same experiment. The geometric mean titres obtained are plotted as a dose response curve in the Figure, and the actual titres obtained with each animal given in Table VII. The results show that with each vaccine some antibody response was produced to each of the three types.

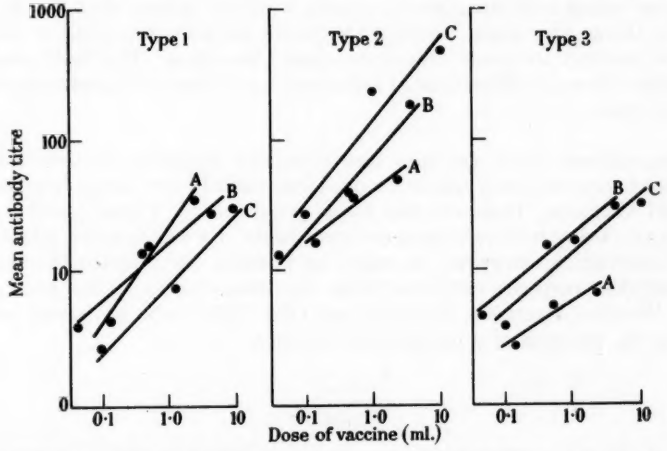

FIGURE.—Neutralising antibody response produced in cotton rats by three trivalent formalinised poliomyelitis vaccines, A, B and C. Two doses were given intraperitoneally, at an interval of 14 days, and the animals were bled 14 days later.

TABLE VII.—*Antibody Titres Produced by Three Poliomyelitis Vaccines in Persons with no Initial Homologous Antibody.*

	Type 1.		Type 2.		Type 3.	
Vaccine.	Number of persons.	Titre.	Number of persons.	Titre.	Number of persons.	Titre.
A	7	43	9	102	13	8
B	15	166	15	147	11	30
C	25	54	27	299	19	48

Titres are given as reciprocals of the geometric mean.

As these vaccines were being used to inoculate human subjects, a few tests were made to determine their antigenicity in man. Since the course of injections varied somewhat within the three vaccines, and as the number of individuals tested who had no initial antibody to any one type of virus was small, it was not possible to make any serious comparison between the response in cotton rats and in man. Nevertheless, it was noted (Table VII) that in man the Type 3 component of vaccine A was the least antigenic and this was reflected to some extent by a very poor response in cotton rats.

DISCUSSION

The Committee of the National Foundation for Infantile Paralysis on Typing of poliomyelitis strains (Report, 1951, 1953) showed that by using monkeys, the poliomyelitis viruses could be divided into three distinct types, but cross neutralisation tests between different strains within each type were not done. Also, the antisera used were produced after more than one injection, which might have tended to obscure any differences that might have been present. Although these factors were taken into consideration here, it would appear that the field strains isolated in Minnesota, when tested with specific antisera produced in cotton rats, showed no marked antigenic difference within the types. This indicates that the poliomyelitis viruses, unlike those of influenza, have been comparatively stable over a period of years.

As formalinised virus vaccines also produced antibody in cotton rats, it is possible that these rodents might be used successfully for comparing the potency of different vaccines. However, the titres produced by Types 1 and 3 after two injections at 14-day intervals were not very high. By varying the schedule or the route of inoculation, however, it might be possible to compare the vaccines by either their dose-response curves or their extinction limits, as has been suggested by Gard, Wesslen, Fagraeus, Svedmyr and Olin (1956), who have used guinea-pigs for testing the potency of poliomyelitis vaccines.

SUMMARY

It has been shown that antibody can be produced in cotton rats by injecting living or formalinised poliomyelitis virus suspensions.

Comparisons by the cross-neutralisation technique of strains isolated in Minnesota over the last few years revealed no difference between strains within each of the three distinct types.

Results suggest that cotton rats might be used for antigenic potency testing of poliomyelitis vaccines.

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QUANTITATIVE STUDIES OF *TOXOPLASMA GONDII* IN CULTURE OF TRYPSIN-DISPERSED MAMMALIAN CELLS

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Toxoplasma gondii cannot be grown in cell-free medium, but multiplies in animal tissues. It is believed that the organism is an obligatory intracellular parasite. It has been shown on many occasions that the parasite enters and apparently multiplies in cells grown *in vitro*; earlier work is mentioned by Chernin and Weller (1954). The cultures were usually prepared by the plasma clot method on slides or in roller tubes, and the cells were derived from tissue of chick embryos except in the work of Lock (1953), who used embryonic rat heart, and Chernin and Weller, who used mouse embryo and human embryo and adult tissues. In these instances the outgrowth of cells would be mainly fibroblastic in type. Cultures of mammalian phagocytes have also been used (Vischer and Suter, 1954; Pulvertaft, Valentine and Lane, 1954). The evidence for multiplication consisted in successful serial passage, and increase in the number of parasites to be counted in the tissue and in the fluid phase of the medium (Chernin and Weller, 1954). Many authors have commented on the fact that heavily infected cells degenerate.

We have carried out studies to demonstrate that the organism multiplies in cultures of normal rabbit and monkey cells and in human malignant cells (strain HeLa). We have obtained quantitative data on the multiplication of the organism in rabbit kidney epithelial cells, and have demonstrated its continued presence in cultures for periods of up to 7 weeks. We have used such cultures as a source of complement-fixing antigen which has been used in diagnostic tests on human and animal sera, and we have shown a marked cytopathogenic effect of the organisms on rabbit kidney cells.

MATERIALS AND METHODS

Toxoplasma

Strain R. H. was passed at 4-day intervals in the mouse peritoneal cavity.

Tissue cultures

All prepared by growing trypsin-dispersed cells directly on glass in stationary bottles or rolled test tubes at 35–36°. When used the bottles had a confluent sheet of cells covering an area of about 50 cm². The methods used have been described (Balducci, Zaiman and Tyrrell, 1956). The medium in rabbit kidney cultures was 5 per cent calf serum, 5 per cent lactalbumen hydrolysate (5 per cent w/v stock solution) and 90 per cent Hanks' solution. Monkey kidney cells were maintained in a similar medium containing 3 per cent calf serum. Cultures of the HeLa strain of human epidermoid carcinoma were maintained in these experiments in 10 per

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cent inactivated horse serum, 10 per cent lactalbumen hydrolysate (5 per cent w/v stock solution) and 80 per cent Hanks' solution.

Staining methods

Permanent preparations of Bouin-fixed roller tube cultures were made by Cheetham's method (Enders and Peebles, 1954) and stained with haematoxylin and eosin.

Complement fixation tests

These were carried out on Perspex plates as described by Fulton and Dumbell (1949) and modified in this laboratory. Antigens were usually titrated against a naturally immune rabbit serum.

Toxoplasma particle counts

Infected fluids were stained with methylene blue and counted in a haemocytometer. These counts were not reliable at levels below 50,000 organisms per ml.

Infectivity titrations

These were carried out by inoculating adult white mice intraperitoneally with 0·5 ml. of the fluid to be tested. Peritoneal washings were taken seven days after inoculation, films made, stained with polychrome methylene blue and examined for parasites under the oil immersion objective. The mouse was regarded as positive if 5 or more organisms were found in 30 microscope fields, but less than 10 per cent of mice which were considered to be infected had as few organisms as this.

EXPERIMENTAL

Infection of Rabbit and Monkey Kidney Cells and Human Carcinoma Cells (Strain HeLa)

Roller tube cultures of rabbit and monkey kidney cells and of HeLa cells were inoculated with 0·1 ml. of 1/10 dilution of mouse peritoneal washings. In each case after 1–2 days degenerative changes were seen on examining the unstained culture (Fig. 2) and permanent preparations made at 4–6 days showed increasing intracellular parasites (Fig. 4 and 5). The changes in unstained cultures began often as localised areas of necrosis with a fringe of granular disintegrating cells which gradually spread to involve more and more of the epithelial cell sheet (Fig. 1 and 3). In rabbit kidney cultures a few cells which appeared to be fibroblastic were not destroyed, and might indeed grow into the space left by the destruction of epithelial cells. Later those too became degenerate. Free toxoplasms were often observed in the medium especially when there was extensive destruction of cells. Three or four serial passages were carried out without difficulty, but cell destruction was less rapid after the first passage.

Toxin

In order to decide whether a toxic principle caused the degenerative changes observed, the following experiment was performed.

A pool of peritoneal exudate from three mice was prepared, diluted 1/100 in culture medium, and divided into five portions. One was heated at 60° in a water-bath for 30 min. Another was alternately frozen and thawed by immersion in a CO_2-alcohol freezing mixture and a 37° water-bath. A third was centrifuged in the Spinco ultracentrifuge at 4° at 7000 *g* for 30 min. The supernatant was used in the experiment. The fourth was filtered through a bacteria-retaining

sintered glass filter (5/3). The fifth was kept as a control. Three 10-fold dilutions of each portion were inoculated in three tubes each. The untreated material caused specific degeneration at a final dilution of 1/1,000, *i.e.*, a 1/10 dilution of the control portion. The treated portions were all non-infective. It is concluded that the degenerative effect observed is associated with the intact parasite and not with a toxin.

Growth and survival of parasites in infected cultures of rabbit kidney cells.

An experiment was done to measure more precisely the production of parasites, and the length of time for which they would persist in the culture.

Six bottle cultures of rabbit kidney cells were prepared. Four bottles were inoculated with 2×10^6 parasites, all four bottles were incubated at 36° and one was removed and placed at 4° after 48 hr. incubation. Two other bottles were inoculated with 2000 parasites, and these were incubated at 36°. The medium was changed twice weekly. In the first 4 weeks half the medium was removed at each change. In addition to this 1-ml. samples were removed on many days to provide material for counting parasites; 1 ml. of new medium was then added after each sample was taken.

The results are summarised in the Figure. The middle panel shows that 7 days after a large inoculum rapid destruction of the culture cells occurred and at the same time numerous parasites appeared in the medium, and the complement-fixation test became positive. After this first phase new cells, which were fibroblast-like, appeared. Their destruction occurred more gradually and was associated with the presence of a small number of parasites in the medium and a negative complement-fixation reaction. At this stage the undiluted culture medium still infected mice, but by the time the fibroblasts were completely destroyed, the infectivity tests and the parasite count were both negative.

The left-hand panel shows that with a smaller inoculum the rise in parasite count and the destruction of cells occurred later, but in other respects the results were identical to those with a large inoculum, except that there was not a long separate phase when only infected fibroblasts were present.

The right-hand panel shows that in a heavily inoculated culture stored at 4° the parasites were gradually released into the medium and could be detected there for 5 weeks. The cultures which were incubated after receiving a small and large inoculum contained mouse-infectious parasites for 6 and 7 weeks respectively.

EXPLANATION OF PLATE

FIG. 1.—Normal rabbit kidney culture. Unstained. × 65.

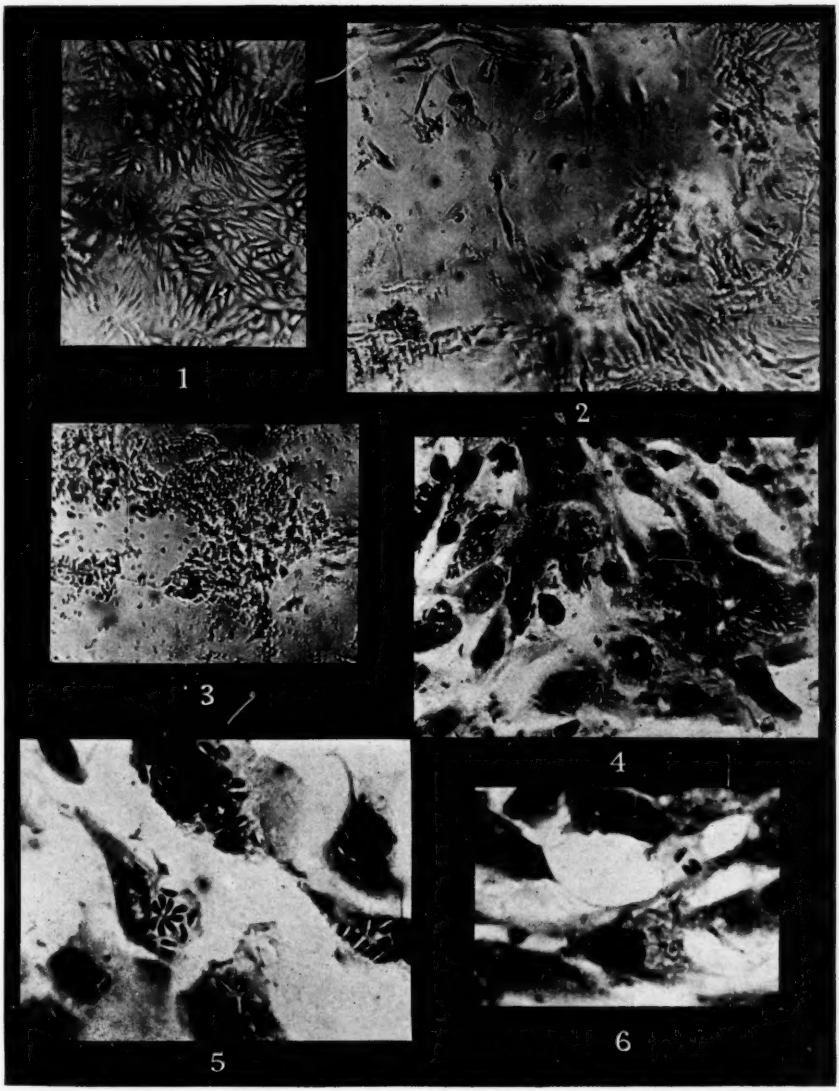
FIG. 2.—Rabbit kidney culture 2 days after infection with toxoplasms. A "hole" in the cell sheet. Unstained. × 100.

FIG. 3.—Rabbit kidney culture 3 days after infection. Many disintegrating cells and extracellular parasites. Unstained. × 100.

FIG. 4.—Monkey kidney culture 6 days after infection. Stained haematoxylin and eosin. × 200.

FIG. 5.—Rabbit kidney culture 2 days after infection, showing "rosette" and "doublet" forms. Stained haematoxylin and eosin. × 200.

FIG. 6.—Rabbit kidney culture 1 hr. after infection. A group of cells showing intracellular parasites: the cultures were washed before fixation. Stained haematoxylin and eosin. × 500.



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Control bottles containing 10 ml. of culture medium but no cells were also inoculated with 500,000 parasites. One bottle was held at 4° after 24 hr. at 37° and the other at 37° throughout. After 1 week there were no mouse-infectious parasites in the medium of either bottle, though parasites were still visible in reduced numbers in the bottle held at 4°.

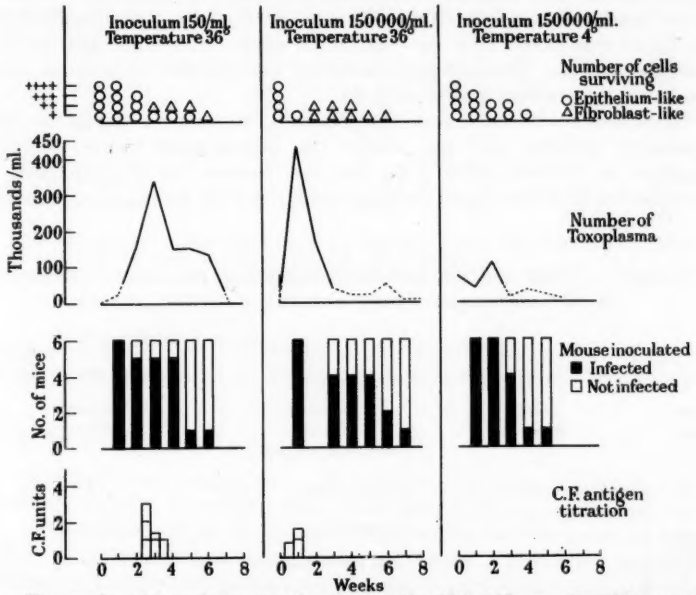

FIGURE.—The results of inoculating bottle cultures of rabbit kidney cells with toxoplasms from mouse peritoneal exudate. The number of cells is indicated by an arbitrary scoring system in which ++++ represents a confluent cell sheet.

We conclude that new toxoplasms are formed in tissue cultures of rabbit kidney epithelium. Complement-fixing activity is only found in fluids containing 300,000 or more parasites per ml. It is of interest that infectious parasites could be recovered up to 7 weeks after inoculation in the fluid phase of cultures of this type. Finally in the bottle held at 4°, the parasites survived up to 5 weeks, presumably in an intracellular site, and were released in a fully active form into the medium. This phenomenon may be parallel to that of the prolonged survival of toxoplasms in hibernating animals, and we would therefore like to name it " artificial hibernation in tissue culture ". This may be a useful practical advance on the preservation of the parasite in extirpated tissues of infected animals, where the maximum survival time observed was 10 days (Kåss, 1955; Cathie and Cecil, 1954).

Complement-fixing antigen from tissue cultures

As has been mentioned earlier, when the fluid phase of tissue cultures contained 300,000 or more parasites per ml. it was possible to show a positive complement

fixation test with immune serum. This test was still positive in some fluids obtained shortly after the parasite count had fallen again below 300,000 per ml. It was a simple matter to produce antigen in useful quantities. Bottle or tube cultures were inoculated with enough parasites to give a final count of $1–2 \times 10^5$/ml. and the fluids harvested when destruction or damage of most cells had occurred. The fluid was frozen and thawed alternately six times, or heated at 56° for 30 min. in order to render it non-infectious. It was clarified by centrifugation at 800 *g* for 10 min. and was stored at 4° or −26° until used. It seemed stable on storage at both temperatures. The antigenic activity was titrated in a chess-board type of test and usually reached a titre of 4–64.

Centrifugation at 10,000 r.p.m. for 30 min. to remove parasites and anti-complementary activity did not affect the complement-fixing antigen titre. Centrifugation at 110,000 *g* for 2 hr. did not reduce the titre further. These results are shown in Table I and indicate that the C.F. antigen is " soluble " and

TABLE I.—*Effect of High Speed Centrifugation and other Treatment on the Complement Fixing Activity of Culture Fluids*

Starting material.	Initial treatment.	Fraction tested.	Units of antigen activity	After centrifugation at: 6,000 *g* 30 min.	After centrifugation at: 110,000 *g* 120 min.
Rabbit kidney culture fluid	56° 30 min.	Not centrifuged	3	—	—
		Supernatant	—	3	2
		Deposit	—	<0·5*	0·3
	6 freeze-thaw cycles	Not centrifuged†	5	—	—
		Supernatant	—	4	—
		Deposit†	—	<0·5	—

* The deposit was re-suspended in 1/7 the original volume and was anti-complementary to a titre of 2 which was the same titre shown in the antigen titration.

† Both original and deposit had an anti-complementary titre of about 2. The supernatant had no anti-complementary activity.

must be of low density or of average density and molecular or macromolecular size. Antigens made from tissue cultures, but not centrifuged at high speeds, were used to titrate positive sera from various sources. The results are shown in Table II. There is correlation between the results of the dye test and the complement-fixation tests, although, as usual, the former is nearly always much higher in titre. There is quite close agreement between the titres obtained by the different antigens. It is also clear that the new procedures we have developed give substantially the same results as the usual ones. The column headed rabbit kidney neutralisation gives the results of tests carried out to try to prevent infection of rabbit kidney cultures by immune sera. An equal volume of a 1/8 dilution of serum was mixed with a toxoplasm suspension which contained 500–600,000 parasites per ml. and held overnight at 4° and 2 hr. at 37°. Of this mixture 0·5 ml. was then added to rabbit kidney tube cultures. In each case toxoplasms

TABLE II.—*Comparison of Results of Serological Tests on Various Sera*

Serum used.	Dye* test.	Rabbit kidney neutralisation.	Complement fixation with antigen from: Chorio-allantoic membrane.†	Tissue culture of: Rabbit kidney.	Monkey kidney.	HeLa.
Human No. 1 412	2600	<16	384	320	320	256
„ 2 363	1800	<16	640	160	256	160
„ 3 766	640	<16	8	16	4	6
„ 4 413	200	<16	>64	64	10	64
„ 5 35	180	<16	8	<4	<4	4
„ 6 SCM	92	<16	<4	<4	<4	4
„ 7 765	80	—	8	<4	<4	6
„ 8 252	43	—	64	32	32	64
Rabbit artificially immune	—	<16	—	32	64	64
„ naturally immune	—	<16	512	512	512	512

* By the method of Sabin and Feldman (1948) and Beverley and Beattie (1952).
† Carried out in tubes by the method of Sabin (1949).
Both these tests were carried out independently in the laboratory of Professor Beattie.

multiplied and caused cell destruction as rapidly as in the control cultures containing serum which had a titre less than 4 in the complement fixation test.

Morphological studies

The morphological appearance of parasites as seen in fixed and stained preparations has been one of the main tools used in studying the growth of toxoplasms. We attempted to study the appearances in the cells as the parasite multiplied. It is possible in tissue cultures to have a relatively uniform sheet of cells uniformly exposed to parasites for a short period and thus to obtain more definite correlation of the appearances with different steps in the process of multiplication.

Cells from infected cultures were used at intervals from 1 hr. to 5 days. In one procedure the cells were scraped from the sides of a tube culture from which free toxoplasms had been removed by washing. The scraped cells were spread on albumenised slides. These preparations were usually fixed with methyl alcohol and stained with Giemsa or Feulgen stain. Other cultures were fixed in Bouin's solution embedded in collodion as described by Cheetham (Enders and Peebles, 1954) and stained with haematoxylin and eosin.

The parasites in these preparations showed the usual morphological features. In one experiment the cultures were infected with mouse peritoneal exudate, and slides fixed 1 hr. later showed that parasites had entered many cells; some parasites were rounded in outline and showed separation of the nuclear material and a slight indentation also present in some suggested that cell division was already in progress (Cross, 1947; Holz, 1954). Parallel parasite counts and infectivity titrations were carried out and indicated that a large fraction of the added parasites had disappeared from the fluid and entered the cells 1 hr. after inoculation. At 1 hr. occasional cells were seen to contain as many as 4–9 parasites. By 4 hr. cells contained 2 and 4 or sometimes more parasites, and some small " rosettes " were visible, such as have been seen in chick embryo

fibroblasts (Mühlpfordt, 1952). By 18 hr. many cells were stuffed with parasites, up to 30 or 40 in number and arranged at times in paired forms (Fig. 5 and 6).

In occasional cells there were many densely stained rather small parasites, (Holz, 1954; Mühlpfordt, 1952) but no pseudocysts were seen. The Giemsa-stained films, especially in the first 4 hr. after infection, showed toxoplasms which looked " faded " and were apparently losing their staining properties (Vischer and Suter, 1954). In later preparations it appeared on comparison with fixed and embedded cultures that the process of making films disrupted most of the heavily infected cells. The films showed many cells containing parasites and some containing coarse granules coloured like the granules of intact parasites. When the inoculum was not infected mouse peritoneal exudate but tissue culture fluid with the same parasite count, far fewer cells in the culture became infected.

DISCUSSION

By techniques similar to those used in virus work we have been able to observe concurrently the destruction of cells and the formation of new parasites and specific antigenic material in cultures of rabbit, human and monkey epithelium, and we think those new hosts for toxoplasms may have practical value in several directions. In the first place they provide a good source of antigen for complement-fixation tests. The parasites persist in an active state for many weeks in cultures held at 4° or at 37° with occasional changes of medium and this may prove a convenient way of preserving a strain without continuous serial passage in animals. It seems that the organism is preserved because of its intracellular site since when suspended in culture medium infectivity disappears rapidly and the number of visible parasites falls.

We have found that infectivity of culture fluids for mice is about 100-fold less than that of mouse peritoneal fluid containing comparable numbers of parasites, and although this could be due to loss of " mouse virulence " it is more probably due to the non-specific loss of infectivity of parasites suspended in culture medium. It is probably because the parasite is unstable in the fluid medium that the infection in cultures seems to spread centrifugally from a small number of infected foci. This type of lesion is characteristic of viruses which spread directly from cell to cell rather than *viâ* the medium (Black and Melnick, 1955).

Our stained preparations show the morphological features of multiplying parasites which others have described. We have also seen the " fading " of some parasites after entering the host cell, although this was not so marked as to suggest that the parasites had " disappeared " from the cells after a few hours, as Vischer and Suter noted. However, we believe that only fully infective active parasites readily enter the epithelial cells of our cultures, and that only a proportion of a suspension of toxoplasms are in this state. As Vischer and Suter used the highly phagocytic macrophage as their host cell it is likely that they were more impressed by the digestion of the many non-viable toxoplasms ingested by these cells than by the survival and rapid multiplication of a smaller number of active parasites the progeny of which became obvious only some hours later. It is clear that the multiplication of toxoplasms can be so rapid in tissue cultures that a light infection can be transformed to a heavy one in a few hours. We saw no reason to postulate a highly toxic form of the parasite distinct from that which multiplied rapidly (Pulvertaft *et al.*, 1954).

SUMMARY

Monolayer epithelial cell cultures derived from rabbit kidney, monkey kidney and human epidermoid carcinoma cells (strain HeLa) can be effectively parasitised and destroyed by *Toxoplasma gondii*. Particle counts and complement fixation antigen titrations show that new toxoplasms are formed while cell destruction occurs.

Tissue culture fluids were used as antigens in complement fixation tests by a micro-method on Perspex plates. The results were comparable with those using conventional procedures, and it was shown that the complement-fixing antigen is non-sedimentible in the ultracentrifuge.

Serial morphological studies showed that cell division in rabbit kidney epithelial cells was a rapid process and that the appearances of the dividing parasites were similar to those seen in other hosts.

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THE MECHANISM OF TOXIC HEPATIC NECROSIS

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THE effect of many noxious influences on the liver is necrosis of the parenchymal cells, but the site of this necrosis varies. Most common around the central vein, it can also occur in the midzone or around the portal tract. The different lesions can be reproduced in animals by special diets or toxic chemicals and these experimental lesions have been widely studied in the hope of determining the mechanism of liver necrosis.

One theory, recently supported by Christie and Judah (1954) and Cameron (1954), attributes the effect of hepato-toxic agents such as carbon tetrachloride (CCl_4) to direct interference with cellular metabolism. Their predilection for certain areas of the lobule could be explained by metabolic differences between the cells of those parts (Mann, 1932 ; Deane, 1944). Another theory, advanced mainly by Himsworth (1947), attributes centrilobular necrosis to ischaemia of the cells around the central vein. Thus, CCl_4 necrosis is centrilobular because the sinusoids are narrowed by swelling of the parenchymal cells which reduces the blood supply to the central cells, whereas after allyl formate these cells are preserved because the sinusoids are dilated and the compound exerts a direct toxic effect on the periportal cells. This view, accepted by Williams (1948), Baxter (1948), Drill (1952) and Elias (1955) to explain toxic centrilobular necrosis, was extended by Himsworth (1947) and Glynn, Himsworth and Lindan (1948) to explain the massive hepatic necrosis and diffuse fibrosis produced by abnormal diets in rats. Glynn (1951), however, admits uncertainty about the mechanism of the initial swelling of the cells after CCl_4 which leads to the circulatory obstruction.

Very different methods have been used in attempts to substantiate these theories. Those seeking a direct effect of toxic chemicals on cellular metabolism have relied on tissue slice techniques or, more recently, on the study of particulate cell fractions. On the whole the chemical findings have been poorly correlated with the histological appearances and there are numerous discrepancies between the reported results. For instance, the O_2 consumption by liver slices from CCl_4-poisoned rats has been described as reduced by Christie and Judah (1954) and Niwa (1954), but as increased by Ennor (1942), Hove and Hardin (1951), Dianzani (1953) and Patwardhan, Ramalingaswarmi, Sriramachari and Patwardhan (1953). Recently a search has been made for specific lesions in the mitochondria. Morphological changes after CCl_4 and phosphorus have been shown by electron microscopy (Dianzani and Bahr, 1954). Dianzani (1954) considers the abnormal mitochondria to behave as if " uncoupling" had occurred. According to Christie and Judah (1954) CCl_4 produces a physical change in the mitochondria destroying their ability to retain necessary co-factors with consequent failure of

energy production. A similar view has recently been expressed by Dianzani (1955). The earliest histological sign of liver damage is in the mitochondria (Cameron and Karunaratne, 1936; Cameron, Milton and Allen, 1943), but it is difficult to decide whether this precedes or follows a vascular change. In either case it is surprising to find an increase in the adenosine triphosphate content of the damaged liver reported by Ennor and Stocken (1948) and confirmed by Rowles (1952).

Vascular changes in the liver during the production of necrosis have been studied in three ways—the distribution of India ink in the liver after injecting it into the spleen, the radiographic observation of the passage through liver of radio-opaque material injected into the portal vein, and direct observation of the trans-illuminated vessels of the liver.

Using the first method, Glynn and Himsworth (1948) claimed that swelling of the parenchymal cells restricted the intralobular circulation as early as 4 hr. after the subcutaneous injection of CCl_4 and that the effect persisted for many hours. Similar results were obtained by Andrews and Maegraith (1948) and by Baxter (1948) in pyridine necrosis although Stowell and Lee (1950) could not detect any difference in the size of the sinusoids in fixed material from CCl_4-poisoned mice. Glynn *et al.* (1948) also reported sinusoidal contraction in diet-induced fatty infiltration of the liver but this could not be confirmed by Fite (1954).

Daniel, Prichard and Reynell (1952*a*), who obtained variable results by the India ink technique, used the radiographic method and could not detect any alteration in the vascular pattern of the liver or rate of portal blood flow in either acute CCl_4 poisoning or fatty infiltration due to a high fat diet.

Loeffler and Nordmann (1925) transilluminated the liver edge in rats and mice exposed to chloroform. They observed constriction of the sinusoids lasting several hours. Wakim and Mann (1942) also found intra-hepatic vasoconstriction by this method, but only so long as CCl_4 was being inhaled. After exposure the normal appearance rapidly returned with later (20–24 hr.) obliteration of the sinusoids as necrosis became obvious. Seneviratne (1949) found only dilatation of the sinusoids during the first 8 hr. after the subcutaneous injection of CCl_4 followed by their compression and ultimate obliteration in the necrotic areas at 24 hr.

These three methods may be criticised for requiring anaesthesia and laparotomy which interfere with the temperature, and blood flow through the liver (Birnie and Grayson, 1952). Furthermore, in the transillumination method observation is confined to the edge of the liver, the behaviour of which may differ from that of the deeper parts (Daniel and Prichard, 1951; Elias, 1955).

The development of internal calorimetry by Grayson (1952) for the simultaneous determination of liver temperature and blood flow in the conscious animal suggested a fresh approach to this problem. It was hoped that by using this method to study the hepatic necrosis produced by a variety of toxic agents, information would be obtained which would help in deciding whether vascular or metabolic factors are more important in its production.

METHODS

Male rats (345 $\pm$ 45 g. body wt.) of the Porton strain fed on M.R.C. diet 41 (Bruce and Parkes, 1949) were used.

Centrilobular necrosis was produced in three ways. Dimethylnitrosamine (DMN), from the sample used by Barnes and Magee (1954), was injected subcutaneously as a 5 per cent (w/v) solution in 0·9 per cent (w/v) NaCl. CCl_4 (Analar) was either injected intra-

muscularly undiluted (Cameron and Karunaratne, 1936) or given by mouth as a 20 per cent (v/v) solution in arachis oil. Na fluoroacetate (Hicks, 1950) was injected intramuscularly as a 0·2 per cent (w/v) solution in 0·9 per cent NaCl.

Beryllium sulphate was injected into a tail vein as a 0·8 per cent (w/v) aqueous solution of $BeSO_4 . 4H_2O$; it produces necrosis which is initially midzonal (Scott, 1948; Aldridge, Barnes and Denz, 1949).

Periportal necrosis was produced by injecting redistilled allyl formate subcutaneously as a freshly prepared 1·5 per cent (v/v) solution in 0·1 M phosphate buffer (pH 7·4).

Recrystallised 3 : 5 dinitro-*o*-cresol (DNOC) (B.D.H.) was also used as a 1·0 per cent (w/v) solution in 0·1 M Na_2CO_3 injected intramuscularly. Ethyl ether (Analar) was injected intramuscularly. All these liquids except the last and allyl formate were warmed to 37° before injection.

The methods of internal calorimetry have been fully described by Grayson and his colleagues (Birnie and Grayson, 1952; Grayson, 1952; Grayson and Haigh, 1953; Grayson and Johnson, 1953; Ginsburg and Grayson, 1954; Johnson, 1954) and were used here with minor modifications (Stoner, 1954). Each recorder was used once and the instrument factor (F) determined in each case (Haigh, 1954). The mean value of F for 153 recorders was 118·2 ± 12·7 (S.D.). Results are expressed as the conductivity increment (δk) due to the flow of blood in the tissue around the recorder. This figure is the difference between the thermal conductivity of the living and dead tissue, measured as the square of the current, in amperes, required to raise the temperature of the heater filament 1° above that of the surrounding liver, divided by F for the recorder. A linear relationship between δk and liver blood flow over the physiological range has been established (Grayson, 1952; Grayson and Haigh, 1954; Grayson and Ginsburg, quoted by Johnson, 1954) even after the recorders have been embedded in the liver for some days (Grayson, 1954). Essentially the method measures the blood flow through a collar of tissue about 2 mm. radius and 2–3 mm. length around the recorder. There may be a considerable error in the "measurement of small percentage changes in flow at low initial blood flow levels" (Johnson, 1954). This observation arose from experiments on severe hypotension after elimination of the portal blood supply to the liver, but with normal initial blood flow levels it is unlikely that a major change in blood flow will be overlooked (Grayson, 1954). Provided that two other requirements are fulfilled the method should be adequate for the present purpose.

The tissue surrounding the recorder must be homogeneous (Grayson, 1952). This has been emphasised by the experience of Linzell (1953). Normal liver meets this requirement and the lesions produced in the present experiments are considered sufficiently diffuse for the method to be valid. Calder (1942) has shown the uniform way in which $CHCl_3$ injures the liver and this has been confirmed for CCl_4 and the other toxic agents in the lobe of the liver used in these experiments.

The successful use of the internal calorimetry method presupposes that the basal thermal conductivity, *i.e.*, of the "dead" liver, does not alter during the experiment (Grayson, 1952). The thermal conductivity values obtained at the end of the experiments show that this assumption is justified (Table I).

TABLE I.—*Thermal Conductivity* (k) *of Rat Liver after Death.*

Treatment.	Number of rats.	$k \times 10^4$ mean ± S.D.
Controls	28	11·8±1·23
Dimethylnitrosamine	24	11·9±0·93
Carbon tetrachloride	13	11·5±1·20
" " + arachis oil	13	11·7±0·96
Arachis oil	12	12·3±1·36
Sodium fluoroacetate	10	10·9±1·01
Beryllium sulphate	10	11·8±1·17
Allyl formate	16	11·6±1·14
Ethyl ether	9	11·2±0·90

The recorders were inserted into the right lobe of the liver (*pars centralis* of Gershbein and Elias, 1954) at laparotomy under ether anaesthesia on the day before the experiment began. After the operation 100 mg. oxytetracycline hydrochloride ("Terramycin—oral

drops "—Pfizer. Oral LD_{50} 720 mg. per 100 g. body wt. (P'an, Reilly, Halley, Richard, Pekich and Pollats, 1950)) were given by stomach tube and this standard dose was repeated each day after the last observation had been made. This prevented peritoneal infection during the period (< 5 days) of the experiment. The dose was based on the experience of Cheng (1954) in this laboratory. It could probably be reduced for the present purpose, but no ill effects were observed. Large doses of oxytetracycline cause liver damage (Lepper *et al.*, 1951), but no evidence of this was found in the controls given this dose. The rôle of infection in liver necrosis is debatable (György, 1951, 1952, 1954; Luckey, Reyniers, György and Forbes, 1954; Reynell, 1954; Eger, 1955), but the presence of an antibiotic in the present experiments did not appear to alter the pathological changes. Lindenbaum, White and Schubert (1954) have shown that only intravenous oxytetracycline chelates effectively with Be. The oral dose used here had no effect on the intravenous toxicity of $BeSO_4$.

The rats were placed in small wire cages (1·5 cm. mesh) while the observations were made. This took 5–10 min. and the animals usually remained quiet during this period. These cages did not restrict respiration and most of the rats could turn round in them. Hypothermia due to restriction and emotion (Ware, Hill and Schultz, 1947; Bartlett, Bohr, Helmendach, Foster and Miller, 1954) was thus avoided.

Each value of δk shown in the text is the mean of 2–4 readings. The liver temperature was measured to the nearest 0·1°.

At the end of the experiment the rat was killed with coal gas if it had survived the action of the toxic agent. After a short time for cooling, the thermal conductivity of the dead liver was measured. The position of the recorder in the liver was then examined and rats in which it was too near the surface or too close to major vessels were excluded. The I^2/θ test (Grayson and Johnson, 1953) was not used routinely, but a sufficient number of tests were made, both before and after death, to show that when the recorder was correctly placed the linear relationship between the square of the current (I) applied and the rise (θ) in temperature produced was unaffected by the poisons used. The recorder was finally removed and the liver around it fixed in Helly's fluid for histological examination.

Where possible results are expressed as the mean $\pm$ S.D. and a statistical comparison of the means made by Student's "t" test as modified by Fisher (1934) for small samples. The illustrations show the results in individual rats chosen because they exhibit most of the features being described.

RESULTS

Preliminary Control Observations

Since the observations usually extended over some days while the hepatic lesion was forming, some preliminary work was necessary to determine the range of the day-to-day variation in the liver temperature and blood flow. Some observations of this type were made by Birnie and Grayson (1952) and Grayson and Johnson (1953) but more detailed information was required for the present work.

Temperature

The mean liver temperature in 176 rats was 38·9° $\pm$ 0·5, in good agreement with the value given by Birnie and Grayson (1952). The mean liver temperature remained very constant over a number of days (Table II) but the temperature was consistently higher in the morning than in the afternoon. The average fall during the day, *i.e.*, between 9 a.m. and 6 p.m., was 0·7° $\pm$ 0·3 in 31 rats observed over a total of 76 days. As this appeared to be part of a diurnal cycle 12 rats were observed at intervals throughout a 24-hr. period. The liver temperature varied in a cyclical fashion (Fig. 1) the sequence of events being as follows.

From 9 a.m. the liver temperature fell steadily during the day while the rat was asleep. Towards evening, when the rat woke up, the temperature rose and reached its first maximum at about 10 p.m. The temperature then fell slightly

TABLE II.—*Mean Liver Temperature and Blood Flow* (δk) *in Rats observed between 9 a.m. and 6 p.m. on Succeeding Days.*

		Day of experiment.			
		1.	2.	3.	4.
I. Fed rats	Liver temp. °C.	38·5±0·3 (23)	38·6±0·4 (23)	38·6±0·3 (17)	38·4±0·3 (9)
	$\delta k \times 10^{4}$*	10·3±3·3 (17)	10·3±3·1 (17)	—	—
		9·5±2·7 (11)	9·5±2·2 (11)	8·8±2·6 (11)	—
		8·2±1·6 (6)	8·1±2·2 (6)	8·0±2·5 (6)	7·7±2·6 (6)
II. Rats fasted after day 1	Liver temp. °C.	38·6±0·3 (7)	38·4±0·4 (7)	38·5±0·4 (6)	—
	$\delta k \times 10^{4}$	8·6±2·2 (5)	8·5±1·3 (5)	7·4±1·6 (4)	—

* The values of δk in each line refer to the values obtained in the same group of rats. Number of rats shown in parentheses.

and later rose to a second maximum at 4–5 a.m. in all but 2 of the 12 rats. The temperature fall during the night was never as great as during the day so that the mean liver temperature between 8 p.m. and 5 a.m., 39·0° ± 0·26, was significantly ($P < 0.01$) higher than the mean day (9 a.m.–6 p.m.) temperature, 38.6° ± 0·27. The first nocturnal peak, 39·3° ± 0·34, was usually higher than the second, 39·0° ± 0·34, but the difference was not significant in this small series ($P < 0.1$; > 0.05).

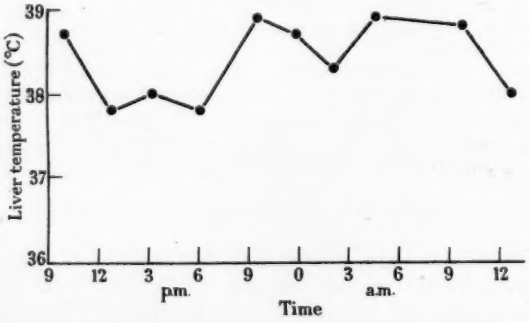

FIG. 1.—The cyclical variation in the liver temperature of a normal rat during a 24-hr. period.

These changes in liver temperature can be related to the diurnal rhythm of the liver constituents and blood sugar in the rat (Higgins, Berkson and Flock, 1932, 1933 ; Deuel, Butts, Hallman, Murray and Blunden, 1938 ; Pitts, 1942). The blood sugar level is highest at midnight, when it is about 10 per cent above the noon level. Similarly the liver glycogen level in the male is highest about

4 a.m. and lowest at about 4 p.m. These changes occur in rats feeding naturally and it can be shown that they are related to the feeding cycle rather than to the activity cycle. The variations in liver temperature, corresponding to changes in its metabolic activity, can also be most readily related to the two periods of food ingestion during the night.

Although these diurnal changes are determined by food intake they are not immediately abolished by fasting. The blood sugar cycle persists during fasts up to 48 hr. (Pitts, 1942). Similarly, the variations in liver temperature were not obliterated by fasts of this duration. This is important, since the food intake of the poisoned rats was reduced. In 7 rats food was removed after the first day's observations and the effect of a 48-hr. fast with free access to water is shown in Table II. The mean liver temperature fell slightly and the temperature fall between 9 a.m. and 6 p.m. was reduced from $0{\cdot}7° \pm 0{\cdot}24$ to $0{\cdot}5° \pm 0{\cdot}21$ but these differences were not statistically significant.

Blood flow

The mean liver blood flow ($\delta k \times 10^4$) from 145 observations on normal rats was $10{\cdot}1 \pm 3{\cdot}4$, again in good agreement with the resting value found for this species by Grayson and his colleagues. As pointed out by Birnie and Grayson (1952) and Grayson and Johnson (1953), the liver blood flow determined by this method shows little day-to-day variation (Table II) and remains very constant in an individual animal (Fig. 2). Very occasionally, after the recorder had been

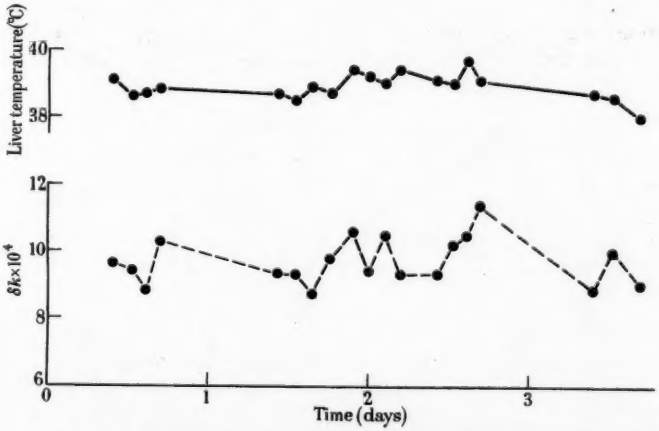

FIG. 2.—The liver temperature and blood flow (δk) in a normal rat over a period of four days.

in place for several days, the value of δk fell to a low level presumably because of fibrosis around the recorder. Temperature and blood flow in the liver are not necessarily correlated (Stoner, 1954) and there was no obvious diurnal variation in the blood flow. In the 12 rats where observations were made at intervals over 24 hr. the mean level of $\delta k \times 10^4$ between 9 a.m. and 6 p.m. was $9{\cdot}3 \pm 3{\cdot}3$ and

between 8 p.m. and 5 a.m. $10{\cdot}1 \pm 4{\cdot}1$, the difference not being statistically significant. The constancy of the liver blood flow during the day was also seen when the initial value of δk in the morning was compared with the mean value obtained during the rest of the day and with the maximum value recorded during that period (Table III). Fasting somewhat reduced the mean blood flow (Table II), but the difference was not statistically significant.

Histology

The appearance of the liver surrounding the wires 2 and 5 days after their insertion is shown in Fig. 3 and 4. There was very little acute inflammatory reaction to the presence of the wires in the liver, but after 3 days fibroblasts could usually be seen proliferating around them. The intensity of the fibroblast reaction varied considerably, but after 5 days the average width of this zone was about 0·3 mm. The parenchymal liver cells around the track appeared normal at all stages.

Metabolic inhibitors

To test this method of assessing the metabolic activity of the liver by determining its temperature the responses to Na fluoroacetate and DNOC were observed. The former should reduce the liver temperature by blocking the

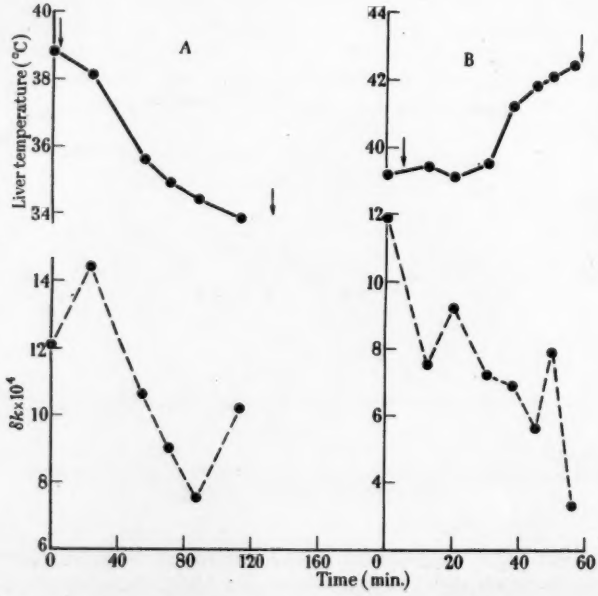

FIG. 5.—The effect of metabolic inhibitors on liver temperature and blood flow (δk) in the rat.
A. 0·5 mg. Na fluoroacetate per 100 g. body wt. injected intramuscularly at the first arrow.
B. 2·3 mg. dinitro-*o*-cresol per 100 g. body wt. injected intramuscularly at the first arrow.
Time of death indicated by the second arrow.

tricarboxylic acid cycle, whereas the latter should raise the temperature by stimulating oxidation without increasing the store of energy-rich phosphates. Fatal doses of these compounds produced these effects (Fig. 5).

" Toxic infarction "

To test the validity of the blood flow measurements under pathological conditions the phenomenon of " toxic infarction " was studied. This is a condition described by Cameron, Karunaratne and Thomas (1937) following the intraportal injection of CCl_4. A sufficiently uniform " infarction " of the liver was produced by injecting 0·1 ml. CCl_4 into as small a tributary of the portal vein as possible (Fig. 6.). The acute effect of this on the hepatic circulation of the anaesthetised rat after laparotomy has been studied by Seneviratne (1949) with the quartz rod technique. He reported a transient fall in liver blood flow with " extreme contraction of the sinusoids, small portal radicles and their terminal branches " lasting for a few minutes. The exactly similar effect on the conductivity increment

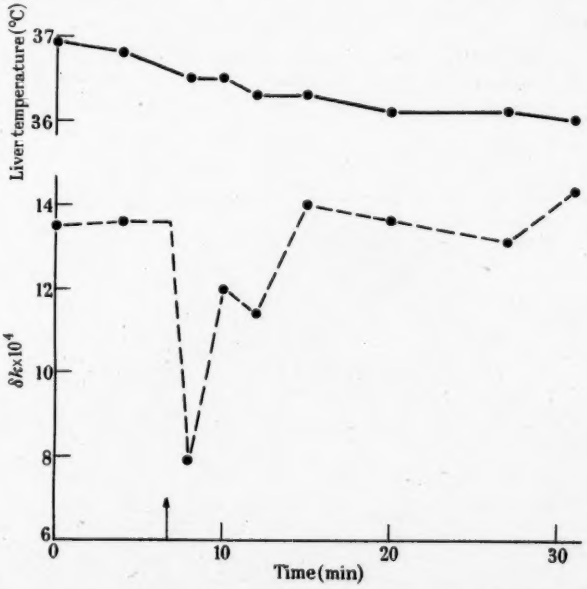

FIG. 7.—The effect of "toxic infarction" on the liver temperature and blood flow (δk) in the anaesthetised (ether) rat, 0·1 ml. CCl_4 injected into the portal vein at the arrow.

(δk) is shown in Fig. 7. The liver temperature underwent no dramatic change after the injection of CCl_4 but fell gradually until the rat was killed, in this case after 45 min. The low initial temperature and its subsequent fall in these experiments can, most probably, be attributed to the anaesthesia (ether), exposure of the liver and gradual deterioration of the preparation. In this instance the results of transillumination and internal calorimetry were in good agreement.

Centrilobular Necrosis

Dimethylnitrosamine $(CH_3)_2N.NO$

This compound was given subcutaneously in doses of 5·0 mg. per 100 g. body wt. This was a certainly fatal dose, the average survival time in those allowed to die being 56 hr.

The microscopical changes in the liver have been described by Barnes and Magee (1954). The first changes are seen after about 6 hr., when there is a decrease in the basophilia of the cells in the centre of the lobule and dilatation of the sinusoids. By 24 hr. there is well marked centrilobular necrosis and, at death, all cellular outline has been lost in the necrotic areas, which are well demarcated from the surviving cells (Fig. 8) and are frequently haemorrhagic.

This dose of DMN produced a very constant change in the liver temperature and blood flow (Fig. 9). During the first 8 hr. after the injection of DMN at about

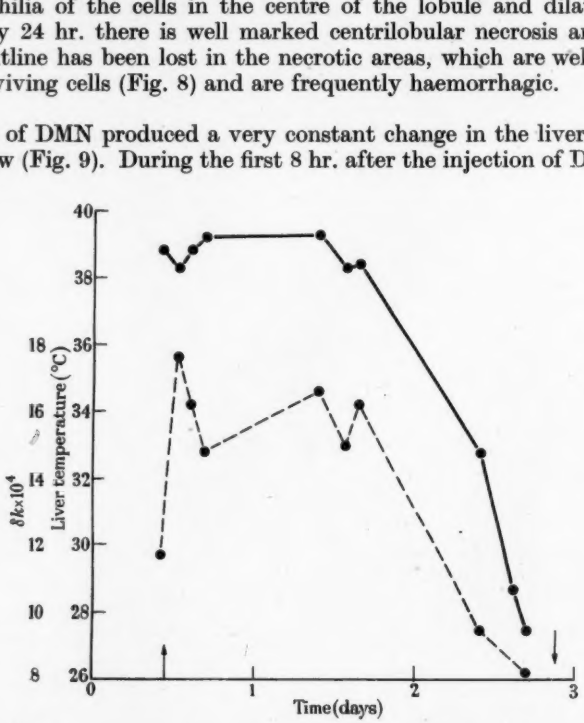

FIG. 9.—The effect of centrilobular necrosis on the liver temperature and blood flow (δk) in the rat. 5·0 mg. dimethylnitrosamine per 100 g. body wt. was injected subcutaneously at the first arrow. Time of death indicated by the second arrow.

10 a.m. on the first day of the experiment the liver temperature fell 0·8° ± 0·29. This was just significantly greater than in the controls. The hepatic blood flow, however, rose. Both the mean blood flow level during the day and the maximum blood flow recorded during that period were significantly higher than the pre-injection level (Table III). The first observations of liver blood flow following the injection of DMN were usually made after about 2 hr. when the rise in δk had usually begun. Some earlier observations were made, but there was no evidence that the rise in blood flow was preceded by a fall. After DMN, δk was

increased in all the rats, but in 2 out of 21 the rise was delayed until 24 hr. after the injection, the blood flow having remained within normal limits during the first 8 hr.

TABLE III.—*Effect of Certain Hepatotoxic Agents on the Liver Temperature and Blood Flow* (δk) *during the First* 24 *hr. after their Administration.*

Compound injected.	Fall in liver temp. between 0 and 8 hr. after injection °C.	Liver blood flow ($\delta k \times 10^4$). Before injection.	0–8 hr. after injection. Max.	0–8 hr. after injection. Mean.	24 hr. after injection.
DMN (5·0 mg./100 g. body wt. subcutaneously)	0·8 ±0·29† (20)	9·6±3·3 (19)	13·1±3·9‡ (19)	12·2±3·8† (19)	12·1±4·3* (14)
CCl_4 (0·1–0·8 ml./100 g. body wt. intramuscularly)	0·9 ±0·5† (14)	12·2±2·9 (12)	14·1±3·2 (12)	13·1±3·0 (12)	12·6±2·6 (12)
$CH_2F.COONa$ (0·2 mg./100 g. body wt. intramuscularly)	5·4 ±1·96§ (10)	10·9±4·6 (10)	11·3±3·9 (10)	10·2±3·6 (10)	10·3±4·3 (10)
$(C_2H_5)_2O$ (0·4 ml./100 g. body wt. intramuscularly)	5·0 ±2·3§ (9)	9·3±3·2 (8)	12·7±7·3 (8)	10·0±3·5 (8)	9·1±2·6 (7)
Controls	0·65±0·3 (76 observations in 31 rats)	9·9±3·3 (20)	10·6±3·5 (20)	9·9±3·3 (20)	9·6±3·0 (20)

Levels of P for the differences from controls (temp.) and from pre-injection level (δk).
* = < 0·10 > 0·05. † = < 0·05. ‡ = < 0·01. § = < 0·001.
Number of rats shown in parentheses.

Twenty-four hours after the dose of DMN the general condition of the rats was still fairly good and in 63 per cent the liver temperature was at its usual morning level but in the remainder it had begun to fall. This fall became general as the rats became ill and once this process had begun it continued until the animal died. Death usually occurred on the third day, when the liver temperature was about 25°.

In 78 per cent of the rats the liver blood flow was still increased 24 hr. after the injection of DMN and in half of these it was higher than on the previous day. In the others δk was about the same as it had been initially. This variability prevented the mean level at this time from showing a statistically significant difference from the mean initial level (Table III). Later, when the liver temperature fell, the blood flow through it decreased in a similar progressive fashion (Fig. 9).

Two additional rats were given 5·0 mg. DMN per 100 g. body wt. by stomach tube. The changes in liver temperature and blood flow were the same as when this dose was given subcutaneously.

Carbon tetrachloride

The histological changes in rat liver after a single dose of CCl_4 have been described by Cameron and Karunaratne (1936). For the first 2 hr. changes are confined to the mitochondria. After 5 hr. there is sinusoidal congestion, but the

parenchymal cells still appear normal. Centrilobular necrosis is present after 24 hr. The central cells show loss of structure, marked eosinophilia and nuclear changes, and these necrotic zones are bordered by the characteristic "balloon" cells produced by this agent (Fig. 10). The doses used here do not produce a fatal lesion; the necrotic cells are removed and regeneration, well established after 3 days, is complete in about 14 days. The study of the changes during the development of necrosis has therefore been concentrated on the events of the first 24 hr.

In the first group of experiments undiluted CCl_4 was injected intramuscularly. To try to find conditions giving a typical response the dose was varied between 0·1 and 0·8 ml. per 100 g. body wt. in 10 rats feeding normally and in 5 fasted overnight. The outcome was uninfluenced by the nutritional state and there was no clear dose-response relationship within that range of intramuscular doses. Accordingly all the animals in which there was histological evidence for necrosis of half or more of the liver lobule (Fig. 10) have been grouped together (Table III).

The fall in liver temperature during the first 8 hr. after the administration of CCl_4 was significantly greater than the fall in the controls during that period. However, at 24 hr. the temperature was the same as it had been before the injection. The liver blood flow was not decreased during the production of the lesions, indeed the tendency was for it to increase although the differences between the means (Table III) were not statistically significant. Confirmation of this tendency was obtained by comparing the differences between the initial and maximum levels in the CCl_4-treated rats with the same differences in the controls. These differences were significantly ($P < 0·02$) greater in the CCl_4-treated rats.

These results agree in part with those reported by Seneviratne (1949). He also found an initial increase in liver blood flow, but described a restriction of the circulation through the lobules when necrosis was established at 24 hr. This difference is of little importance from the point of view of the mechanism of the necrosis and the production of a more severe lesion in the presence of greater increase in liver blood flow will now be described.

CCl_4 is more effective when given by mouth (McCloskey and McGehee, 1950). In a second group of experiments CCl_4 was given by this route as a 20 per cent (v/v) solution in arachis oil, the dose being equivalent to 0·2 ml. CCl_4 per 100 g. body wt. Because Stohlman (1948) has shown that oral doses of corn and olive oils lower the rectal temperature the results were compared with those in controls given the same amount of arachis oil by mouth.

Oral doses of arachis oil affected both the temperature and blood flow of the liver (Table IV, Fig. 11). The liver temperature fell rapidly so that the reduction during the first 8 hr. was about twice that in untreated controls (Table III). At the same time δk was significantly increased. Twenty-four hours later normal values were obtained for the temperature and blood flow of the liver.

Addition of CCl_4 to the arachis oil greatly increased the immediate fall in liver temperature without altering the increased liver blood flow (Table IV). The temperature depression produced by 0·2 ml. CCl_4 per 100 g. body wt. given by mouth as a 20 per cent solution in arachis oil was greater than the sum of the separate effects of that amount of arachis oil by mouth and the same dose of CCl_4 intramuscularly. The fall in liver temperature was also more prolonged than after either substance alone (Fig. 11). Twenty-four hours after the administration of CCl_4 in arachis oil the liver temperature had regained its pre-injection level in only 2 of the 14 rats. By this time the δk level was the same as in the controls

TABLE IV.—*Effect of Carbon Tetrachloride in Arachis Oil and Arachis Oil alone on the Liver Temperature and Blood Flow* (δk) *during the First 24 hr. after Oral Administration.*

Compound injected.	Fall in liver temp. between 0 and 8 hr. after injection °C.	Liver blood flow ($\delta k \times 10^4$). Before injection.	0–8 hr. after injection. Max.	Mean.	24 hr. after injection.
Arachis oil (1·0 ml./100 g. body wt. p.o.)	1·5±0·6 (12)	9·5±3·7 (12)	13·0±4·0† (12)	11·7±3·5 (12)	10·2±4·0 (12)
CCl_4 (0·2 ml./100 g. body wt. 20% (v/v) solution in arachis oil p.o.)	2·9±0·8* (14)	9·7±3·2 (13)	13·4±4·6† (13)	11·9±4·3 (13)	10·1±3·9 (13)

* = significantly different from "arachis oil" controls at $P < 0·001$.
† = significantly greater than the pre-injection level at $P < 0·05$.
Number of rats shown in parentheses.

(Table IV) and normal temperature levels were found after 48 hr. The further progress of these rats was not studied. Histologically, they showed much more severe hepatic necrosis than those given the same dose of CCl_4 intramuscularly despite the greatly increased hepatic blood flow during the development of the lesion.

Sodium fluoroacetate (*$CH_2F.COONa$*)

Although best known as an inhibitor of the tricarboxylic acid cycle (Peters, 1951), fluoroacetate in small doses which are not immediately fatal will produce

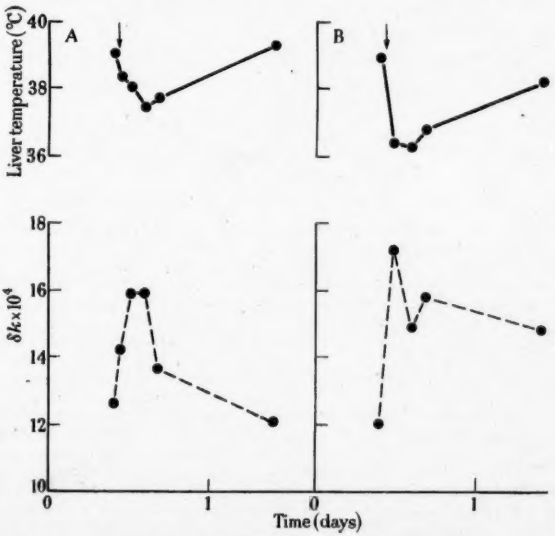

FIG. 11.—A comparison of the effects of oral doses of arachis oil with and without CCl_4 on the liver temperature and blood flow (δk) of the rat. A. 1·0 ml. arachis oil per 100 g. body wt. given by mouth at the arrow. B. 0·2 ml. CCl_4 per 100 g. body wt. given by mouth at the arrow as a 20 per cent (v/v) solution in arachis oil.

centrilobular hepatic necrosis (Hicks, 1950). The effect of rapidly lethal doses on liver temperature and blood flow has been described above. For the present purpose 0·2 mg. Na fluoroacetate per 100 g. body wt. was injected intramuscularly. This dose, close to the M.L.D., produced occasional convulsions, but all the 10 rats used survived more than 24 hr. after its injection. All showed centrilobular necrosis, but in comparison with the other agents used this was never severe (Fig. 12).

Liver temperature and blood flow were observed for the first 24 hr. after the injection of fluoroacetate (Table III). The liver temperature fell steeply so that after 3¼ hr. it was 4–5° below the pre-injection level. In 7 of the 10 rats it was still depressed after 24 hr. and in 4 of these the liver temperature was still falling. Despite this striking effect on liver temperature there was no definite alteration in liver blood flow (Table III) although in individual rats the fluctuations were often greater than in the controls.

"*Midzonal*" *Necrosis*

"Midzonal" necrosis of the liver was produced by the intravenous injection of 0·11–0·15 mg. Be^{2+} per 100 g. body wt. This dose, twice the LD_{50} (Aldridge *et al.*, 1949), was usually fatal, the survival time being about 58 hr. The histological changes in the liver were similar to those described by Scott (1948) and by Aldridge *et al.* (1949). With this dose there was definite zonal hepatic necrosis after 24 hr. From being initially a zonal lesion the necrosis spread so that at death practically all the liver cells were affected (Fig. 13).

Be^{2+} in a dose of 0·11–0·15 mg. per 100 g. body wt. produced very constant changes in the liver temperature and blood flow (Fig. 14). Almost immediately (10 min.) after the injection 80 per cent of the rats showed an increase in δk. This was not usually maintained and, as it was sometimes seen after the intravenous injection of equivalent volumes of 0·9 per cent NaCl, may well be non-specific.

EXPLANATION OF PLATES

All sections (5μ) stained with haematoxylin and eosin.

FIG. 3.—Section of the liver of a control rat cut parallel to the recorder wires after they had been in place for two days; shows the central track left by the wires and normal liver parenchyma on either side of it. (× 50.)

FIG. 4.—Section of the liver of a control rat cut at right angles to the recorder wires after they had been in place for five days. There is some fibroblast proliferation around the site of the wires with normal liver parenchyma beyond. The diameter of the black circle corresponds to 4 mm., being the diameter of the cylinder of tissue in which the blood flow is measured. (× 17.)

FIG. 6.—"Toxic infarction." Section of the liver of a rat killed 45 min. after the injection of 0·1 ml. CCl_4 into a small branch of the portal vein under ether anaesthesia. (× 50.)

FIG. 8.—Centrilobular necrosis. Section of the liver of a rat dying 54 hr. after the subcutaneous injection of 5·0 mg. dimethylnitrosamine per 100 g. body wt. The section has been cut at right angles to the recorder, the position of which is shown by the central space. (× 50.)

FIG. 10.—Centrilobular necrosis. Section of the liver of a rat killed 24 hr. after the intramuscular injection of 0·2 ml. carbon tetrachloride per 100 g. body wt. The section has been cut at right angles to the recorder, the track of which is seen in one corner. (× 50.)

FIG. 12.—Centrilobular necrosis. Section of the liver of a rat killed 24 hr. after the intramuscular injection of 0·2 mg. Na fluoroacetate per 100 g. body wt. This rat showed the most severe hepatic lesion produced by this agent. (× 100.)

FIG. 13.—"Midzonal" necrosis. Section of the liver of a rat dying 55 hr. after the intravenous injection of 0·15 mg. Be^{2+} per 100 g. body wt. The section has been cut at right angles to the recorder, the track of which is seen in one corner. (× 100.)

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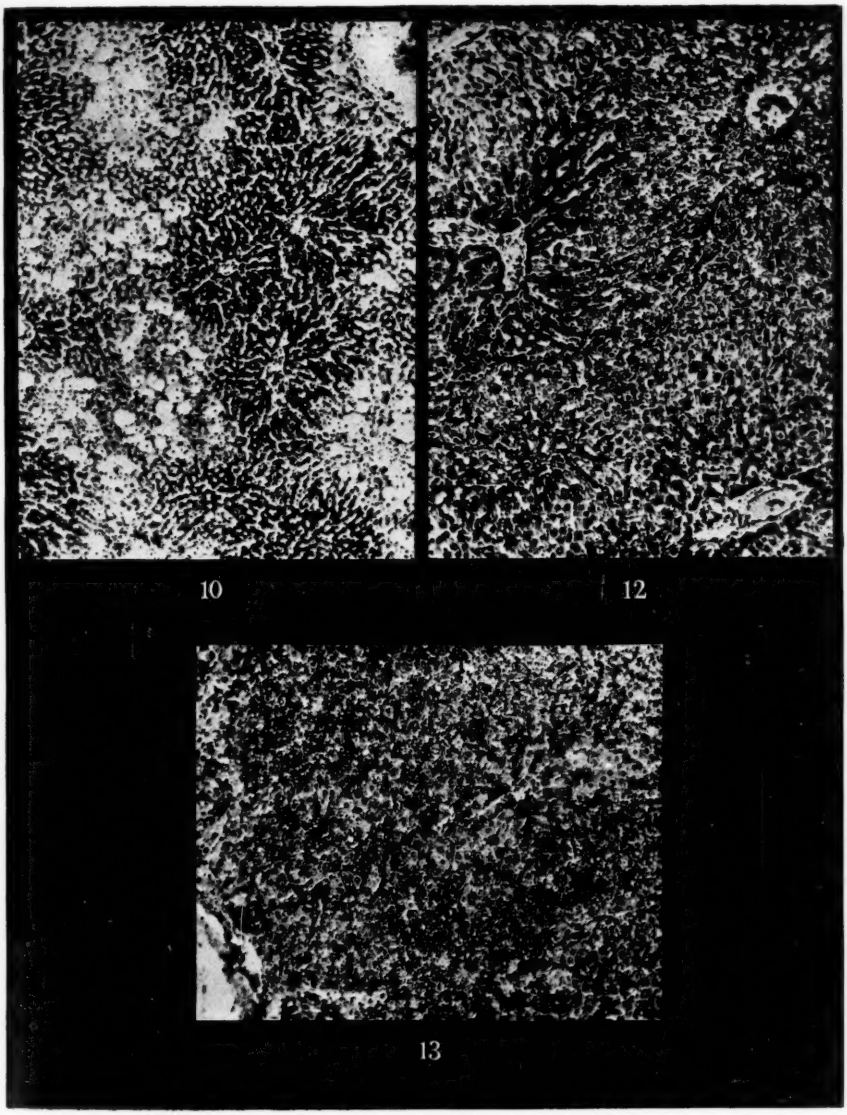

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The blood flow changes in the first hour after Be have therefore been excluded from the statistical examination of the δk values (Table V), which shows that there was no significant change in δk during the first 32 hr. after the injection of Be.

TABLE V.—*Effect of* 0·11–0·15 *mg.* Be^{2+}/100 *g. body wt. intravenously on the Liver Temperature and Blood Flow* (δk) *in the Rat.*

Liver temperature °C.			Liver blood flow ($\delta k \times 10^4$).		
Mean fall 0–8 hr. after Be.	Mean temp. 0–8 hr. after Be	Mean temp. 24–32 hr. after Be.	Before Be.	Mean 1–8 hr. after Be.	Mean 24–32 hr. after Be.
0·7±0·35 (14)	38·9±0·29 (14)	37·9±0·62* (14)	9·5±2·7 (9)	10·7±2·1 (9)	9·4±3·2 (9)

* = Significantly different from the mean temp. 0–8 hr. after Be at $P < 0{\cdot}001$.
Number of animals shown in parentheses.

There was no immediate effect on liver temperature, its fall during the first 8 hr. (Table V) being the same as in the controls. The temperature was still well maintained on the day after the injection, but at a lower level than before. Comparison of the mean temperatures during these periods showed a significant difference and this drop in liver temperature after 24 hr., corresponding to the appearance of zonal necrosis, was the first sign of damage due to Be. Subsequently, with the increasing necrosis, the liver temperature fell progressively as the rat became moribund. This was accompanied by a reduced blood flow, but it was noticeable that there was no appreciable change in δk until there had been a marked fall in liver temperature (Fig. 14). In this the Be-treated rats differed from those given DMN for in the final stage of that illness liver temperature and blood flow fell together. The character of the change in hepatic blood flow was the same as

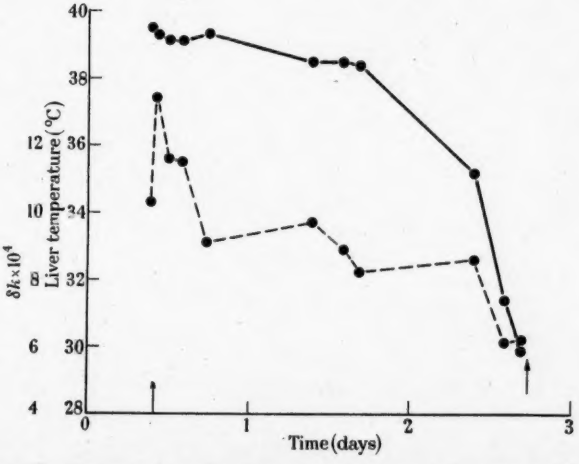

FIG. 14.—Effect of " midzonal " necrosis on the liver temperature and blood flow (δk) in the rat : 0·15 mg. Be^{2+} per 100 g. body wt. was injected intravenously at the first arrow as an aqueous solution of $BeSO_4$. Death occurred at the second arrow.

that described by Cheng (1956) using the quartz rod and flash photomicrographic techniques. He found a progressive contraction of the sinusoids beginning after 3 hr. with extensive sinusoidal obliteration after 16 hr.

Periportal Necrosis

Allyl formate (*$HCOO.CH_2CH : CH_2$*)

Allyl formate has been commonly used to produce periportal necrosis since its properties were first noted by Piazza (1914). Eppinger (1937) used it in the development of his concept of " serous inflammation ", for he considered that it was primarily a vascular poison and that the periportal lesion was secondary to this. Heinemann (1937) maintained that allyl formate did not produce hepatic necrosis in the rat, but it would seem from the work of Rosin and Doljanski (1946) and from the present experiments that her results were due to an unfortunate choice of doses.

TABLE VI.—*Average Survival Time and Histological Picture after Intramuscular Doses of Allyl Formate in the Rat.*

Dose. ml. per 100 g. body wt. intramuscularly.	Survival time (hr.). Mean ± S.D.	Histology.
0·003	Indefinitely	Congestion of the sinusoids with increased eosinophilia and some pyknosis of the nuclei in the parenchymal cells around the portal tracts. Signs of recovery after 48 hr.
0·006	21·3±12·8	Oedema of the portal tracts with an excess of inflammatory cells. Necrosis of the periportal cells seen as early as 8 hr. after the injection and in rats surviving more than 24 hr. formed a band about four cells wide. Beyond this zone there was an area of increased cytoplasmic eosinophilia and vacuolation.
0·008 0·009	5·6± 1·9 5·4± 1·5	More severe changes were seen at an earlier stage. There was marked eosinophilia, pyknosis and loss of nuclei with areas of coagulative necrosis occupying up to three quarters of the liver lobule.
0·015	1·5± 0·4	—

The dose used varied between 0·003 ml. and 0·015 ml. per 100 g. body wt., the M.L.D. for this strain of rat being about 0·005 ml. per 100 g. body wt. The average survival time and the histological changes, in haematoxylin and eosin preparations, following the different doses are given in Table VI. For any given dose the histological changes were more variable than after the other compounds used. The speed with which periportal necrosis developed after the larger doses was very remarkable and was accompanied by equally dramatic changes in both liver temperature and blood flow.

The most constant effect of the injection of allyl formate was a fall in liver temperature (Fig. 15), and after the larger doses this continued until the rat died. After 0·003 ml. allyl formate per 100 g. body wt. the fall continued for about 2 hr., after which it slowly recovered so that normal values were obtained on the following day. This effect was so constant that when the mean fall in liver temperature during the first 2 hr. was plotted against the logarithm of the dose a clear log. dose-response relationship was seen for the dose range (0·003–0·009 ml.

per 100 g. body wt.) in which observations were made (Fig. 16). With all the doses the fall in temperature was significantly greater ($P < 0{\cdot}001$) than in the controls.

The changes in liver blood flow were more complex. The percentage change in δk 2 hr. after the injection of allyl formate varied both in size and direction, and there was no dose-response relationship (Fig. 16). Results in individual rats showed that the predominant effect was a fall in δk. After fatal doses the hepatic blood flow was invariably decreased shortly before death, usually having fallen steeply, as in Fig. 15A, to very low levels. This was not their initial effect, however, for there was nearly always a period commencing about 30 min. after the injection

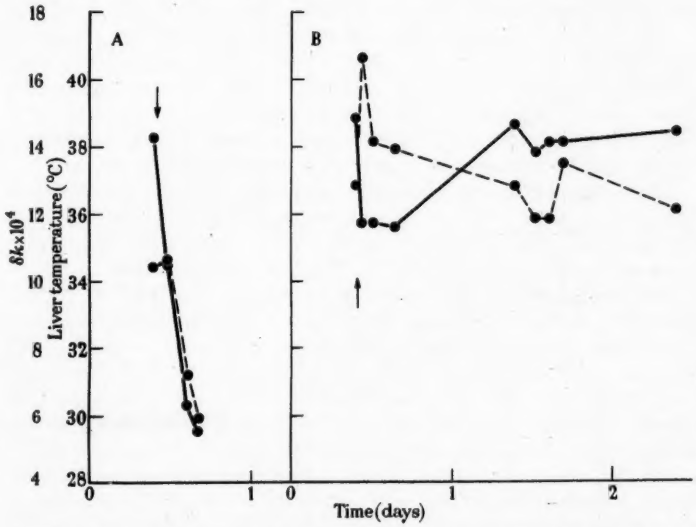

FIG. 15.—Effect of periportal necrosis on the liver temperature and blood flow (δk) in the rat. A. 0·006 ml. allyl formate per 100 g. body wt. injected intramuscularly at the arrow. The rat died during the night. B. 0·003 ml. allyl formate per 100 g. body wt. injected intramuscularly at the arrow.

and lasting 1–2 hr. when the blood flow was increased. This was a transitory effect, usually complete by 2 hr. after the injection, which appeared to correspond to a phase of generalised vasodilatation, for the ears showed a pink or purplish flush at that time. Since the liver temperature fell *ab initio* this means that the temperature had fallen several degrees (Fig. 15 and 16) before there was any significant fall in liver blood flow. This dissociation of the temperature and blood flow effects was best seen in rats given 0·003 ml. allyl formate per 100 g. body wt. These animals showed a proportionate fall in liver temperature (Fig. 16), but the change in δk was usually confined to the transitory rise, after which the blood flow remained within normal limits (Fig. 15). Very little necrosis, at the most a few scattered foci, was seen in rats given this dose. The characteristic necrotic lesion was only seen after the larger doses, which depressed both the temperature and the blood flow.

The Effect of Ethyl Ether

To obtain further control data the effect of ethyl ether was observed since it does not cause hepatic necrosis yet has pharmacological properties in common with $CHCl_3$ and CCl_4.

Rats were given 0·4 ml. ether per 100 g. body wt. intramuscularly. This dose did not produce complete anaesthesia, but made the animals stuporose for 1–2 hr. All except 1 of 15 rats recovered. Histological examination of the liver taken from rats killed 24 hr. after this dose of ether showed congestion of the sinusoids.

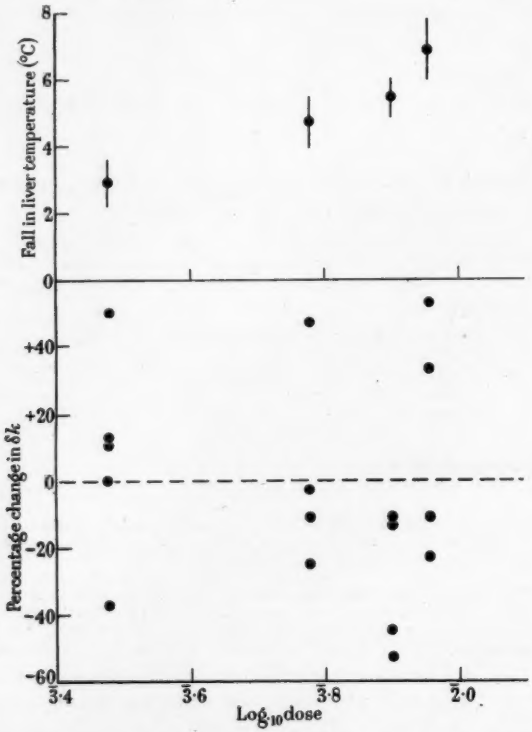

FIG. 16.—The upper part of the Figure shows the fall in liver temperature during the first 2 hr. after different doses of allyl formate in the rat. The points correspond to the mean values and the standard deviation is indicated by the vertical line. The lower part of the Figure shows the percentage change in δk for the liver 2 hr. after different doses of allyl formate. Each point corresponds to an individual rat.

There were numerous polymorphs in the sinusoids and blood vessels and in the track of the recorder. This was probably due to a general leucocytosis in response to the tissue damage at the injection site, for the leg was oedematous and the muscle showed a patchy necrosis. No definite hepatic necrosis was seen. Occasionally the cell and nuclear outlines were blurred in a zone about one cell thick around some of the centrilobular veins.

The changes in liver temperature and blood flow are summarised in Table III. A fall in liver temperature was constant, and except in the rat which died the minimum was reached after about 2 hr., a gradual rise to normal following up to 24 hr. The changes in δk were not so constant. In 4 rats it rose significantly, in 2 it fell significantly and remained unchanged in the remaining 2. These changes cancelled out in the averages (Table III) which showed no significant deviation. In the rat which died the liver temperature and δk fell progressively.

DISCUSSION

If these results can be accepted as indicating true changes in liver metabolism and blood flow it should be possible to draw some conclusions about the mechanisms involved in the production of the lesions studied.

Previous workers are agreed that the temperature of the liver is conditioned by its metabolism, and this view is strongly supported by its diurnal fluctuation, which can be easily related to biochemical events in the liver and by observing the predicted effects of DNOC and fluoroacetate.

Grayson and his colleagues have shown that an accurate picture of the changes in liver blood flow during physiological experiments can be obtained by internal calorimetry. It is unlikely that a major change in blood flow, such as might occur under pathological conditions, would be overlooked, particularly as the lesions did not alter the basal thermal conductivity of the liver or the I^2/θ relationship. The results of internal calorimetry cannot be directly correlated with those of dye injection or transillumination since each method measures a different aspect of blood flow. Many workers have found the simple dye injection method unreliable. In its most refined form as used by Daniel *et al.* (1952*a*) it gives information mainly about the structure of the blood vessels and the rate of flow in them, as for instance in these authors' study of the cirrhotic liver (1952*b*). The work most relevant to the problem of hepatic necrosis comes from the use of the transillumination technique and some attempt at correlation must be made. At first, when a direct comparison with transillumination was made under similar conditions of anaesthesia and laparotomy, *i.e.*, in toxic infarction, closely similar results were obtained. Later, discrepancies appeared which must be considered. Two features of internal calorimetry should be re-emphasised at this point. Firstly, it assesses the blood flow through a small collar of tissue around the recorder and the effects observed are due to changes in those cells. Secondly, it does not record the flow at any instant but the mean flow during the period required to take a reading, about 1 min.

The position of the recorder in the depths of the liver may explain why no evidence was found, either in the controls or in the rats given toxic agents, of the occasional restriction of the circulation through the superficial regions of the liver described by Daniel and Prichard (1951) using a radio-opaque dye and seen by most users of the transillumination technique. Other factors may be involved in this, for both methods differ from internal calorimetry in requiring anaethesia and laparotomy during the experiment. These factors may explain the other discrepancies. It would seem from the results of Seneviratne (1949) with CCl_4 and of Cheng (1956) with Be compounds that the transillumination method is more sensitive than internal calorimetry. However, it may be that the circulation in the damaged liver is more sensitive than in the normal liver to the depressant

effects of anaesthesia and laparotomy. This idea is supported by the protocols shown in Table VII. It is thought that by the elimination of these factors internal calorimetry may give the most faithful picture of the changes in liver blood flow.

TABLE VII.—*Effect of Nembutal and Laparotomy on the Liver Temperature and Blood Flow* (δk) *in a Rat given* CCl_4.

	Liver temp. °C.	Liver blood flow ($\delta k \times 10^4$).
I. CCl_4-treated rat :		
Before CCl_4	39·4	8·3
24 hr. after CCl_4	36·4	8·1
After operation	34·2	3·1
II. Control (no CCl_4) :		
Before operation	38·9	9·9
After operation	36·1	9·7

CCl_4 was given in a dose of 0·2 ml. per 100 g. body wt. by mouth as a 20 per cent solution in arachis oil 24 hr. before operation.

The immediate effect of all the compounds causing centrilobular necrosis was accentuation of the day-time fall in liver temperature, indicating depression of the liver metabolism. To this extent the results support the " metabolic " theory of the production of centrilobular necrosis. However, although with CCl_4 the greater the fall in temperature the more severe the necrosis, among the different compounds there was no correlation between the magnitude of this effect and the size or histological appearance of the lesion. Fluoroacetate with the greatest effect produced least necrosis. The marked effect of this compound on liver temperature was expected, since it gives rise to a potent inhibitor of cell oxidation. DMN, with little effect on the O_2 uptake of liver slices (Vandekar, personal communication), only slightly increased the fall in temperature yet produced the most severe lesion in the liver. The necrosis produced by DMN was fatal and towards the end both liver temperatures and blood flow fell dramatically. Necrosis preceded these terminal changes, which might reflect either the final failure of the liver or the moribund state of the rat. These alternatives could not be differentiated and both factors may be involved.

These experiments on centrilobular necrosis give no support to the "ischaemia" theory of its production. With all the agents studied the hepatic blood flow was either unchanged (fluoroacetate) or increased (DMN and CCl_4) during the production of the lesion. The increase was most marked after DMN and with the particular compounds used was positively correlated with the severity of the lesion. With CCl_4 the more severe lesion was produced in the face of the large increase in blood flow due to the arachis oil. There is no reason to suppose that these changes in δk have arisen other than by the action of these compounds on the liver tissue around the recorder.

The centrilobular necrosis produced in these experiments cannot be attributed to circulatory disturbances and is thought to be due to direct interference with the metabolism of the affected cells. It is not possible from these experiments to say much about the metabolic reactions concerned. It is clear that centrilobular necrosis was not due to simple interference with the oxidative mechanisms of the affected cells. This is shown by the lack of correlation between the fall in liver temperature produced by a compound and its necrotic action and also by the fact

that ether markedly lowers liver temperature without producing necrosis. The nature of the primary biochemical disturbance, which may be different in each case, and the possibility that the localisation of the lesion is due to biochemical differences between the central and peripheral cells await further study.

A possible explanation of the " midzonal " necrosis produced by Be compounds has recently been proposed by Cheng (1956) working in this laboratory. He considers that after injection Be circulates as a colloidal compound which is removed by the Kupffer cells. These undergo necrosis and this process spreads, with diffusion of the toxic agent, to the adjacent liver cells to give the histological appearance seen after about 24 hr. The circulatory disturbance in the liver is thought to be secondary to these changes. The results obtained with internal calorimetry fit Cheng's theory very well. The latent period before significant change in liver temperature agrees with the idea that alterations in the liver parenchyma only occur after destruction of the Kupffer cells, for these must contribute very little to heat production by the liver. The secondary nature of the decrease in liver blood flow is clearly shown by this method.

Neither of the two explanations (Eppinger, 1937 ; Himsworth, 1947) proposed for the periportal necrosis produced by allyl formate is in complete agreement with the results of internal calorimetry. Allyl formate is certainly more than a vascular poison. This is shown by the log. dose-response relationship for the depression of the liver temperature, and these results support the view of Fleckenstein (1944) and of Kessel, Körtge and Pezold (1954) that it is a general metabolic poison. Indeed, this would seem to be its main action, since the fall in liver temperature is independent of any changes in blood flow. These latter are complex, being the result of opposing forces. While it would be unwise to say that the changes in liver blood flow are entirely secondary to the alteration in its metabolism, the fall in blood flow probably follows the general metabolic depression. If the dose of allyl formate is sufficient this effect interferes with the primary rise in flow. This primary rise seems to be associated with a generalised vasodilator action of the compound. It is, however, doubtful if the dilatation of the sinusoids can be considered to protect the central cells for, after necrotising doses of allyl formate, the flow through them is very slow. On the other hand, does the fall in blood flow help to produce the periportal lesion, for definite necrosis was only seen when this occurred? These data do not provide a simple answer, but the metabolic effects of allyl formate seem to outweigh the vascular ones and it appears reasonable to attribute the periportal necrosis to them.

The general conclusions from this study of the effect of a range of hepatotoxic agents on the temperature and blood flow of the liver are that the lesions they produce are due to interference with the metabolism of the affected cells and that any changes in blood flow are either secondary to this or due to other causes.

SUMMARY

The changes in liver temperature and blood flow during the production of hepatic necrosis by dimethylnitrosamine, carbon tetrachloride, sodium fluoroacetate, beryllium sulphate and allyl formate have been followed in the rat by the method of internal calorimetry to determine whether this necrosis is due to an alteration in the blood supply to the liver cells or to direct interference with their metabolism.

The suitability of the method was tested by observing the effect of 3,5-dinitro-*o*-cresol and sodium fluoroacetate on liver temperature. This was increased by the first and reduced by the second compound in accordance with their known metabolic effects. The changes in liver blood flow in " toxic infarction " produced by the intraportal injection of carbon tetrachloride agreed with Seneviratne's results using the transillumination method.

In the normal rat the liver blood flow remained relatively constant but the temperature of the liver showed a diurnal cycle.

Substances causing centrilobular necrosis immediately increased the day-time fall in liver temperature. This effect was minimal with dimethylnitrosamine and maximal after fluoroacetate. The liver blood flow during the production of the necrosis was unchanged by fluoroacetate but increased with the other compounds. The necrosis produced by the doses used was in the order dimethylnitrosamine > carbon tetrachloride > sodium fluoroacetate.

Fatal doses of beryllium sulphate had no immediate effect on liver temperature or blood flow but after 24 hr., when necrosis was present, the temperature in the liver fell followed by a reduction in its blood flow.

The main effect of allyl formate was on the liver temperature, which fell in proportion to the log. dose over the dose range used. Small doses increased the blood flow, but with larger doses there was a secondary fall in liver blood flow which, after high doses, frequently replaced the early increase.

The intramuscular injection of ethyl ether caused a marked fall in liver temperature without producing necrosis.

These findings are considered to support the view that the lesions of toxic hepatic necrosis are not due to interference with the blood supply to the liver but result from a direct interference with the metabolism of the liver cells.

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HEPATIC CLEARANCE OF BILIRUBIN AS AN INDEX OF CELLULAR FUNCTION IN THE REGENERATING RAT LIVER

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WHEN two-thirds of the rat's liver is removed surgically the remainder increases rapidly in weight so that, after three days, it has almost doubled its mass and 100 per cent of the original weight of the liver is achieved in two weeks. This process has excited considerable interest and studies have been made of the anatomical changes occurring in the remnant (Brues, Drury and Brues, 1936), the factors controlling this growth process (Mann and Magath, 1922 ; Cameron, 1935 ; Mann, 1940, 1944 ; Weinbren, 1953, 1955) and the changes in chemical and enzymic constituents (Stowell, 1948 ; Harkness, 1952 ; Ferrari and Harkness, 1954). Assessment of the functional capacity of regenerating liver has remained a difficult problem, since the reserve power of the liver can seldom be estimated by the clinical liver function tests. Recently, however, the principle of clearance techniques, as applied to renal function, has been adapted for the estimation of the functional activity of the liver (Mason, Hawley and Smith, 1948 ; Lewis, 1950). In these methods the load imposed on the excretory mechanism of the liver is so great that the measurement of clearance is an indication of maximal excretory capacity.

It seemed to be of interest to estimate the actual quantity of a substrate excreted into the bile of rats under various experimental conditions and to find if this procedure could be used to assess the functional capacity of the liver during the process of regeneration after partial hepatectomy. We chose as a substrate bilirubin, intubated the bile duct and then estimated directly the quantity of bile pigment excreted when the level of bilirubin in the blood was such that the excretory mechanism was saturated throughout the period of the experiment.

EXPERIMENTAL

Rats.—Male white rats weighing 220–500 g. were kept on " Research " rat cubes supplemented with white bread and greenstuff. The rats were not fasted before any of the experimental procedures.

Bilirubin.—The priming solution was prepared by dissolving without heating 100 mg. Bilirubin (B.D.H.) in 50 ml. of an isotonic solution containing 0·5 g. Na_2CO_3 and 0·52 g. NaCl per 100 ml. (1 ml. ≡ 2 mg. bilirubin). The solution was freshly prepared for each set of experiments and was kept in the dark. The bilirubin for infusion was prepared by diluting the priming solution with an equal volume of 0·85 per cent NaCl (1 ml. ≡ 1 mg. bilirubin).

The total bile pigments in serum were estimated by the method of Malloy and Evelyn (1937). For the determination of the pigments in bile the volume of the aqueous solution was made up to 10 ml. and a suitable aliquot (0·2–1·0 ml.) was taken and the volume made

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up to 1·0 ml. with water. Volumes of 1 ml. diazo reagent and 8 ml. methyl alcohol were added and after 30 min. readings were made against water on a Beckman spectrophotometer at 540 mμ. Preliminary experiments showed that normal rat bile at these dilutions contained negligible amounts of chromogenic material so that no allowance for endogenous bilirubin was made.

Measurement of maximal excretion of pigment

Veterinary Nembutal (Abbott) was injected intraperitoneally in doses of 0·07 ml./100 g. body wt. with a maximum of 0·21 ml. The common bile duct was cannulated 1 cm. proximal to the duodenum with the shaft of a 24-gauge subcutaneous needle, which had been broken from its base and fitted into a 10–15 cm. length of polythene tubing of 0·5 mm. internal diameter. The tubing was filled with distilled water and its free end was kept under water in a collecting tube at approximately the same level as the common bile duct. The receiving tube was changed every 3 or 10 min., according to the requirements of the experiments.

A priming dose of bilirubin was injected into the left superficial femoral vein and the delivering needle then connected to a syringe driven by an electric motor at a speed of delivery of approximately 1 ml. of solution per 3½ min.

Partial hepatectomy

The left lateral and anterior median lobes (approximately two-thirds) were removed under anaesthesia (Brues *et al.*, 1936).

The preliminary experiments were designed to establish a standard procedure for the estimation of maximal bile pigment excretion in normal rats.

Effect of single injection of bilirubin on serum levels and pigment excretion in the bile

Single doses of 1 mg., 2 mg., and 4 mg. bilirubin per 100 g. body wt. were given each to two rats. The bile duct was cannulated before the injection, blood samples were taken from the right external jugular vein at approximately 5 min. intervals and the bile was collected continuously, the collecting tube being changed every 3 min. Fig. 1 shows that after single injections of bilirubin the maximal pigment output in the bile was attained with a dose of 2 mg./100 g. body wt. The maximal excretory level in the bile was reached about 10 min. after the highest serum bile pigment concentration. In order to assess the serum level necessary for maximum biliary secretion the values approximately 10 min. before maximum bile pigment excretion had to be considered. It was concluded that a serum bile pigment concentration greater than 15 mg. per cent was necessary.

Maximal hepatic bile pigment excretion in normal rats

This was estimated in 16 rats. A priming dose of 2 mg./100 g. body wt. was injected and a continuous infusion delivered at a rate of approximately 1 mg. bilirubin per 3½ min. Bile collections were made over 10-min. intervals from 15–45 min. after administration of the priming dose. At the end of each experiment the liver was removed for weighing and the rat was killed with ether. Similar results were usually obtained from the three 10-min. periods. The final figure for each rat was a mean of the 10-min. figures. The maximal hepatic clearance by this method was 61 μg./min./100 g. body wt. ± S.D. 8·0 (Fig. 2). The mean blood level of the bile pigment at the end of the experiment was 19 mg. per cent. Although the tissues were deeply pigmented at death the quantity of pigment lost in the urine was insignificant.

Experiments in Rats with Regenerating Livers

The following experiments were designed to compare the maximal capacity to clear bilirubin in normal rats with that in rats with regenerating livers. Reference to data from large numbers of partially hepatectomised rats of the same strain confirmed previous observations that the liver remnant increases in size most rapidly during the first three days after operation, and measurements were made therefore during this post-operative period. At the end of each experiment the liver remnant was weighed.

Maximal bile pigment excretion immediately after partial hepatectomy.—Partial hepatectomy was carried out on six rats under light ether anaesthesia. In two control rats laparotomy was performed, the hepatic ligaments were divided, and the liver lobes were mobilised but

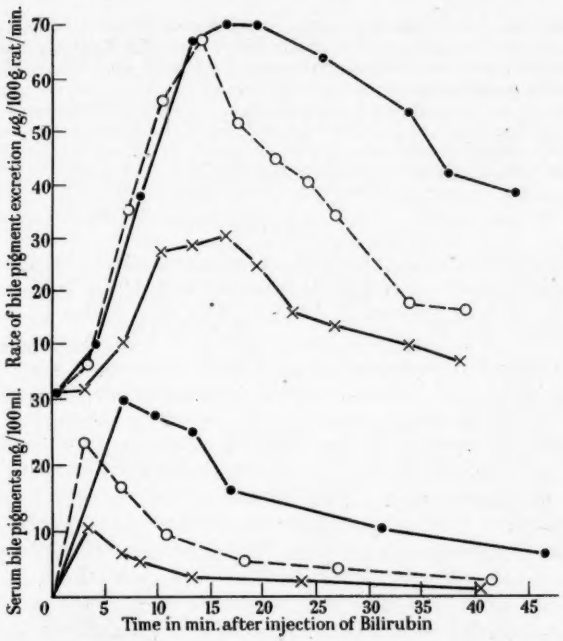

FIG. 1.—Rates of bile pigment excretion after the injection of varying doses of bilirubin.

Amount of bilirubin injected :

× ——— × 1 mg. bilirubin/100 g. rat

○ ——— ○ 2 mg. „ „ „

● ——— ● 4 mg. „ „ „

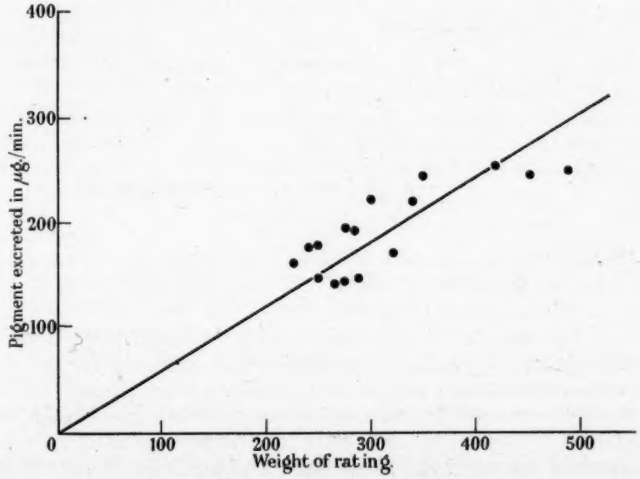

FIG. 2.—Maximal pigment excretion in normal rats.

not resected. By this procedure the control rats were subjected to a comparable degree of anaesthesia and traumatic handling of the liver. Immediately after the operation the animals were anaesthetised with intra-peritoneal Nembutal and the maximal bile pigment excretion estimated as in normal rats.

Maximal bile pigment excretion 24, 48 *and* 72 *hours after partial hepatectomy.*—This was estimated in seven rats 24 hr. after partial hepatectomy and four controls after laparotomy; in eight partially hepatectomised rats and two controls 48 hr. after operation; and in nine partially hepatectomised rats and two controls 72 hr. after operation.

RESULTS

The mean bile pigment excretion, in μg. pigment/min./100 g. body wt., was 18 ($\pm$ S.D. 4·6) immediately after partial hepatectomy, 19 ($\pm$ S.D. 3·6) after 24 hr., 36 ($\pm$ S.D. 7·9) after 48 hr. and 38 ($\pm$ S.D. 6·8) after 72 hr.

For the purpose of comparison these values have been expressed as percentages of the control values for the immediate and 24 hr. post-operative experiments, and as percentages of normal values for later post-operative experiments when the effect of the simple laparotomy appeared to be negligible. Immediately after partial hepatectomy the clearance is 30 per cent of controls, after 24 hr. 31 per cent, after 48 hr. 58 per cent and after 72 hr. 61 per cent. At the same time, the weights of the residual liver tissue have been expressed as percentages of the total hepatic weight, as estimated from the weights of resected lobes (Fig. 3.). In sixteen

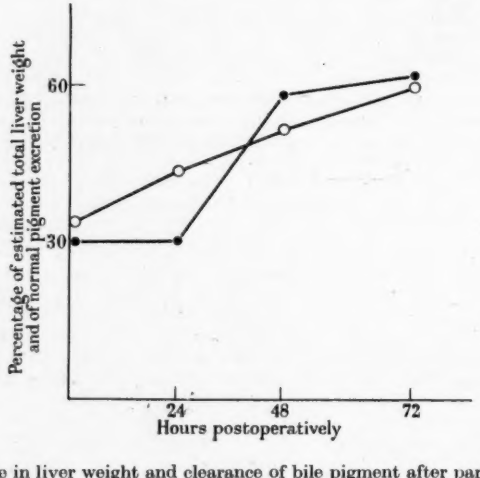

FIG. 3.—Increase in liver weight and clearance of bile pigment after partial hepatectomy.
● Clearance of bile pigment; ○ weight of liver.

control rats of the same strain the relationship between total liver weight and anterior lobes was 1·52 ($\pm$ S.D. 0·1) and this value was used for the estimation of the total liver weight from the weight of the anterior lobes (Brues *et al.*). The mean weight of the liver was 43 per cent of estimated total liver weight 24 hr. after partial hepatectomy, 51 per cent after 48 hr. and 59 per cent after 72 hr. Function is thus diminished for the first 24 hr., but later the pigment excretion tends to increase with the weight gain.

DISCUSSION

The method described in this paper appears to give a reliable estimate of hepatic function, as judged by the ability of the liver to clear bile pigment. The values for the injected dosage of pigment found necessary for maximal clearance agree substantially with the level of 2·2 mg./100 g. body weight found by Hanzon (1952). The clearance of bile pigment under these conditions is much greater than that calculated for basal levels, but both maximal clearance and basal rate of flow may be correlated with body weight (Friedman, Byers and Michaelis, 1950; Cook, Beach, Bianchi, Hambourger and Green, 1950). The method involves direct measurements of the quantity of pigment in the bile and thus determines the amount which is removed exclusively by the liver, and possible extra-hepatic routes of clearance do not complicate the findings. Taleisnik (1955), using bromsulphthalein and applying clearance formulae to the serum concentrations, achieved fairly consistent results but raised the possibility of error in such determinations. The present procedure is not complicated by intestinal re-absorption of the bile pigment as this was avoided by the technique of cannulation.

With regard to regenerating liver tissue, the capacity to clear bilirubin appears to lag for the first 24 hr. This is of interest in view of the findings of Greenbaum, Greenwood and Harkness (1954), who pointed out that the mitochondrial enzymes increase rather more slowly than other cell components for the first 24 hr. after partial hepatectomy. During this early period, Brues *et al.* calculated that the cells of the remnant increased in size but not in number. A further complicating factor in the first 24 hr. is the change in the character of the portal blood flow post-operatively. Daniel and Prichard (1951) claim that the circulation is restricted to the hilar regions, although other workers consider that the flow is increased after partial hepatectomy (Mann, 1940, 1944; Child, 1954). We cannot exclude the possibility that alterations in the hepatic blood flow may have affected the early post-operative values.

The increase in hepatic clearance of bile pigment 48 and 72 hr. after partial hepatectomy suggests that recently formed liver cells are as capable of extracting and secreting bile pigment as older liver cells. This finding is based on a quantitative test in which the principle of saturation of the excretory mechanism has been employed, so that all the liver cells in the remnant have taken part in the excretory process. Our findings are in agreement with the impression that regenerating cells function as well as mature cells in the human (Cameron, 1954), but it is difficult to make a direct comparison with disease processes because of the complications of inflammation, architectural alteration and species differences.

SUMMARY

Maximal hepatic clearance of bilirubin was measured in normal rats and in rats with regenerating livers by infusing bilirubin intravenously and estimating the quantity of bile pigment excreted in the bile.

For the first 24 hr. after partial hepatectomy the excretory capacity does not increase as rapidly as the relative weight of the regenerating liver tissue, but thereafter the excretory capacity tends to increase with the weight increase.

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LABORATORY STUDIES OF APC AND INFLUENZA C VIRUSES

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A NEW group of viruses has been discovered in the U.S.A. by two groups of workers. Rowe, Huebner, Gilmore, Parrott and Ward (1953) found that tissue cultures of surgically removed human adenoid or tonsil often degenerate owing to the multiplication of a virus present in the original tissue. The virus cannot be demonstrated in ground tissue or in swabs from the throat. However, inoculation of fluids from the degenerated adenoids into other human or animal cell cultures gives rise to degenerative cellular changes which can be transmitted serially. Hilleman and Werner (1954) used human epithelial cell cultures and recovered similar viruses from throat washings and sputum from patients (U.S. servicemen) with acute respiratory disease (A.R.D.) or with primary atypical pneumonia. Antibody responses were also found in these patients, thus indicating that these viruses might cause human disease. Serological studies made retrospectively by Ginsberg, Badger, Dingle, Jordan and Katz (1955) on specimens from volunteers to whom A.R.D. was transmitted also indicated a relation between the new group of viruses and human respiratory disease.

Huebner, Rowe, Ward, Parrott and Bell (1954) have shown that there are at least six serological types of these viruses as judged by virus neutralisation tests employing tissue cultures and immune sera prepared in rabbits. They have given the group the name of APC viruses (adenoidal-pharyngeal-conjunctival), and they regard Hilleman's strains as forming one type, namely type 4, of the group; physical properties characterising the group were also described. Others have pointed out that there are minor differences in biological and physical characteristics between certain strains thus far studied of types 1, 2, 3 and 4 (Ginsberg, Dingle and others, personal communications). Huebner *et al.* have isolated many strains of these viruses and typed them, and these studies suggest further differences between different antigenic types. Types 1 and 2 are often isolated from tonsils and adenoids, and so also, though less commonly, is type 5. Type 4 has been mainly found in washings from cases of A.R.D. or atypical pneumonia in servicemen, and type 3 has been found in cases of pharyngitis, often accompanied by conjunctivitis, in civilians, especially children (Parrott, Rowe, Huebner, Bernton and McCullough, 1954; Bell, Rowe, Engler, Parrott and Huebner, 1955).

Strains of all the different antigenic types share a common complement-fixing antigen. This antigen is not deposited by high-speed centrifugation which separates the virus particles, thus suggesting that it exists in a smaller molecular phase (Rowe, Huebner, Hartley, Ward and Parrott, 1955; Hilleman, Tousmis and Werner, 1955). However Pereira (personal communication, 1955) found that

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complement-fixing activity in preparations of the viruses was apparently retained by a collodion membrane just able to retain virus particles.

In a study of material, including faeces, from cases of aseptic meningitis and mesenteric lymphadenitis, Kjellén (1955) isolated agents of this group and showed corresponding antibody rises in the patients' sera.

In order to get some idea of the prevalence of infection with these viruses, antibodies against them have been estimated in a few population groups in the U.S.A. (Huebner *et al.*, 1954; Hilleman, Werner, Dascomb and Butler, 1955). There has been one very brief published report so far of the recovery of a virus of this group in England (Pereira and Chapronière, 1954).

This paper describes studies undertaken in Northern England in 1954 and 1955. The objects of the investigation were, firstly, to recover and identify viruses of the APC group in material collected in this area, secondly, to test sera from various local populations for antibodies against these viruses, and thirdly, to seek cases of disease with specific antibody responses against viruses of this group. Preliminary results of these studies have been published (Zaiman, Balducci and Tyrrell, 1955).

MATERIALS AND METHODS

Tissues

Adenoids.—Obtained at operation and kindly supplied by Mr. J. D. Gray, F.R.C.S. Immediately after removal they were placed in containers of sterile saline containing penicillin and streptomycin, and transported on ice to the laboratory. They were thoroughly washed in several changes of sterile saline containing antibiotics, and chopped into fragments which were set up in roller tube plasma-clot cultures.

HeLa cells.—Cells of strain HeLa, originally derived by Gey from a human epidermoid carcinoma of the cervix in 1951, and propagated since then in continuous culture on glass (Scherer, Syverton and Gey, 1953) were obtained from the Virus Reference Laboratory, Colindale, and grown in continuous culture.

Human embryos.—These were obtained at hysterotomy, and transported in sterile containers to the laboratory. Tissues required were dissected out after washing in several changes of sterile saline. They were then chopped and used for either plasma-clot or trypsin-dispersed monolayer cell cultures.

Human kidney.—Some specimens of human kidney removed at operation were put in containers of sterile saline containing antibiotics and transported to the laboratory on ice. Healthy-looking pieces of cortex were carefully dissected free and chopped. The fragments were used to set up trypsin-dispersed monolayer cell cultures.

Culture Media

All ingredients for tissue culture media were stored in hard or neutral-glass containers at − 26°, unless otherwise stated.

Horse serum.—Burroughs Wellcome Normal Horse Serum No. 2, inactivated at 56° for 30 min. before use.

Calf serum.—Clotted calf blood was obtained from the abattoir, and the serum was filtered through a Sterimat pad* under positive pressure.

Rabbit serum.—Blood was obtained by cardiac puncture or from the lateral ear vein. Serum was separated by centrifugation and did not usually require filtration.

Human serum.—This was kindly supplied by the Sheffield Regional Blood Transfusion Service. Pools were made of samples from not less than three individuals, and filtered through a Sterimat pad under positive pressure.

Bovine amniotic fluid.—Prepared as described by Enders (1953).

Beef embryo extract, chick embryo extract and hen plasma.—Prepared and stored by methods similar to those described by Robbins, Weller and Enders (1952).

* Bacteria-retaining filter pad manufactured by T. B. Ford Ltd., Loudwater, Bucks.

Hanks' balanced salt solution.—Prepared and stored as described by Weller, Enders, Robbins and Stoddard (1952).

Lactalbumin hydrolysate stock solution (5 per cent).—Prepared as described by Melnick and Riordan (1952), stored at 4° and used within 4 days of preparation.

Trypsin.—Difco Trypsin was used, either " Bacto-Trypsin " or " Difco 1 : 250 " made up to a 0·5 per cent solution in Hanks' balanced salt solution (without bicarbonate). This was sterilised by filtration (through a sintered glass bacteria-retaining filter or a Sterimat pad) and stored at − 26°. Before use an equal volume of Hanks' solution with bicarbonate was added to produce a 0·25 per cent solution at a suitable pH.

Methods of Tissue Culture

Culture vessels

Rimless Pyrex test-tubes 16 × 150 mm. closed with tightly-fitting natural rubber stoppers were used. Various flat-sided bottles of about 200–500 ml. capacity and made of good quality glass were used. All glassware was sterilised by hot air at 180° for 2 hr. All media were made up to contain about 50 units of penicillin and 50 μg. of streptomycin per ml. Incubation was at 36–37°.

Plasma clot cultures.—Used mainly for adenoid tissue and were prepared as described by Robbins *et al.* (1952).

HeLa cell cultures

These were grown directly on glass. Stock cultures were maintained in stationary bottles, in a medium consisting of 20 per cent pooled filtered human serum, 10 per cent stock lactalbumin hydrolysate solution (giving a final concentration of 0·5 per cent) and 70 per cent Hanks' balanced salt solution. When tube cultures were required, cells were scraped from the side of the bottle with a rubber " policeman ", pipetted up and down vigorously to break up the larger sheets and clumps and transferred to a graduated centrifuge tube. They were washed three times in new medium, then re-suspended in 5 ml. of new medium containing horse serum instead of human serum. A drop of the suspension was transferred to a haemocytometer and the cells were counted. Extra medium was added to reach the required cell concentration of between 50,000 and 100,000 per ml. Of the final cell suspension 0·5 ml. was put in tubes which were incubated in stationary racks. The tubes were ready for use in 2–5 days, depending on the number of cells introduced. When the cells had flattened out and multiplied to form confluent sheets, the fluid was changed to maintenance medium. In this medium they remained healthy for 6–7 days without a change. We have used many media for maintenance of HeLa cells, but the most satisfactory has been 5 per cent rabbit serum, 5 per cent stock lactalbumin solution and 90 per cent of Hanks' solution with three times the usual concentration of bicarbonate.

Trypsin-dispersed monolayer cell cultures

This method was used for cultivating a variety of tissues, including human embryo lung, human embryo kidney, human kidney, monkey kidney, rabbit kidney and chick embryo lung. All these tissues prepared by this method produce a growth of epithelial-like cells. The technique used was an adaptation of Youngner's modification of Dulbecco's method for the preparation of monolayer cultures of monkey kidney cells (Youngner, 1954). The method of preparation of rabbit kidney is given in full.

Rabbit kidney

Well washed fragments of rabbit kidney cortex (chopped preferably with sharp knives into pieces approx. ½–1 cm. square) were incubated at 37° for 10 min. with 10 ml. of 0·25 per cent trypsin solution per kidney. The supernatant fluid was then discarded, and a further volume of trypsin solution, 10 ml. per kidney, was added. The mixture was stirred gently for 5–6 min. The supernatant containing free cells was decanted through three layers of sterile gauze into chilled containers. More trypsin was added, the mixture was again stirred for 5–6 min. and the supernatant treated as before. This was repeated 8–10 times, or until the kidney fragments remaining were too small to make further " extractions " worth while. The filtered cell suspension was stored at 4° until the extraction procedure was finished. The cells were

centrifuged at 1000 r.p.m. for 5 min., and then re-suspended in Hanks' balanced salt solution containing 5 per cent horse serum. They were washed at least three times in this fluid, being centrifuged each time at 600 r.p.m. for 2–3 min., and being gradually transferred to fewer containers, until finally the cells were all in one or two graduated centrifuge tubes. They were then re-suspended in growth medium for a last washing, and centrifuged again to estimate the packed cell volume. The cells were re-suspended in growth medium at a dilution of 1/50. The suspension was then diluted with further growth medium to contain a final dilution of the packed cell volume of 1/100 to 1/200. (This contained approximately 600,000 to 1,000,000 cells per ml.) The cell suspension was distributed into tubes, 1 ml. in each, and these were incubated in stationary racks. Medium was changed after 6–7 days or earlier if there had been rapid metabolism which lowered the pH below 7·0. Confluent sheets of cells were usually seen at 10–14 days, and before use the growth medium was replaced by maintenance medium.

Similar methods were used for other tissues although small alterations in detail were necessary to get good results. For example, it was necessary to roll human embryo lung cultures from the time they were set up if epithelial rather than fibroblastic outgrowth was to be obtained.

Chick embryo lung

Cultures were prepared as described by Tyrrell (1955) with the exception that the cell concentration used was adjusted to 1/100 to 1/200 of the packed cell volume, corresponding to a cell count of 4–2,000,000 per ml.

Permanent stained preparation.—Made in collodion films (Enders and Peebles, 1954).

Neutralisation Tests in Tissue Culture

Tube cultures of rabbit kidney epithelial-type cells were used. These were easily prepared, and were available in larger numbers than cultures of HeLa cells (which have been used by other workers).

Reference strains of APC types 1–6, and type-specific immune sera prepared in rabbits, were kindly supplied by Dr. H. Pereira. Pools of each type virus were prepared by inoculating tube or bottle cultures of HeLa cells, and harvesting cells and fluid when all the cells had fallen off the glass. These pools were stored at − 26°.

For typing, unknown strains were inoculated into cultures of HeLa cells. Cells and fluid were harvested when all the cells had fallen off the glass, and stored at − 26° until used. In typing tests, unknown virus fluids were used in a final dilution of 1/20, and type-specific antisera were used in a final dilution of 1/20 or 1/30.

In neutralisation tests for the estimation of serum antibody levels against the six available types of APC virus, the dose of each virus used was the highest dilution of the seed which would produce a marked cytopathogenic change in 2–3 days in cultures of rabbit kidney inoculated with virus only. It was found that although pools of each virus were prepared in a similar way, they did not all produce cytopathogenic changes in rabbit kidney to the same degree. Higher concentrations of types 3 and 4 than of the other types were required to produce recognisable changes. Therefore lower dilutions of types 3 and 4 were used in preparation of inocula for neutralisation tests. Sera were usually diluted in fourfold steps, and two or three tubes were inoculated with a serum-virus mixture containing each dilution. Since a large quantity of serum is necessary for tests against each of six types of APC virus, specimens were often inadequate for use in an initial dilution lower than 1/16. Where an adequate amount of serum was available, the lowest initial dilution (before adding virus) was 1/8, and sometimes 1/4.

All dilutions were made in maintenance medium, and all dilutions and mixtures were made up so that the required inoculum was contained in 0·5 ml. Serum-virus mixtures were left at room temperature for 1–1½ hr. before inoculation.

Pairs of sera from cases of acute respiratory illness were always titrated on the same day, in tubes from the same batch of rabbit kidney cells. Control tubes, inoculated with each virus type only, or with medium only, were included in each batch of tests. Cultures were examined at least three times during the course of the test, after 1, 2–3 and 6 days. A cytopathogenic change was often noted in some tubes, which was non-progressive for the duration of the test, but affected fewer cells than in the virus-inoculated controls (scored as + instead of + + +), *i.e.*, there was no all-or-none effect as is usually observed in neutralisation tests in HeLa cells.

However there was a sufficiently clean-cut difference from tubes showing a + + + degenerative effect to allow an estimation of a serum " partial protection " end-point, which was usually 2- or 4-fold higher than a " complete protection " end-point.

Complement Fixation Tests

Tests were carried out using apparatus and procedure similar to that described by Fulton and Dumbell (1949). Serial dilutions of serum were used with a single dilution of antigen containing an " optimal fixation unit " which was determined in " checkerboard " experiments with positive human serum. Fixation was carried out at 4° for about 18 hr. wth 2–2½ units of complement. Sensitised cells (0·4 per cent) were prepared using a final dilution of 1/200 of Burroughs Wellcome haemolytic serum. One drop was added and the test was read after 1½ hr. at 35–36°.

The titres of most of the positive sera were checked by at least one repetition of the test. The maximum disagreement observed was fourfold.

Antigens for Complement Fixation Tests

Influenza A and B.—The strains used were WS and Lee. Eleven-day embryonated hen's eggs were inoculated in the allantoic cavity and incubated for 48 hr. at 35°. Chorio-allantoic membranes were harvested, pooled, and frozen and thawed six times without grinding. The extract was centrifuged at 3500 r.p.m. for 15 min. The supernatant was titrated against a human serum of known antibody titre and stored in small volumes at − 20°.

Influenza C.—Strain 1233 (kindly supplied by Dr. F. L. Horsfall) was used. Eggs of 12–13 days were inoculated intra-amniotically, incubated for 48 hr. at 35°, then chilled overnight at 4°. Amniotic fluids were harvested and titrated against hyperimmune ferret serum and then stored at − 20°.

APC viruses.—Two strains (HA12 and HA13) isolated in Sheffield from adenoids were used. HeLa cell cultures in tubes or bottles were washed free from human serum with three changes of Hanks' solution and maintenance medium was added. Undiluted culture fluid was then inoculated. The cultures were incubated at 36° for 5–7 days, although frequently the cells had all degenerated and became detached from the glass after 3–4 days. Fluids and cells were harvested, frozen and thawed one or more times, homogenised, and centrifuged. The supernatant was titrated against a human positive serum, kindly supplied by Dr. M. R. Hilleman, then stored in small volumes at − 20°.

Human sera.—Clotted venous blood was obtained by aseptic methods from patients and volunteers. Either immediately or after being sent to the laboratory by post the serum was separated and stored at − 20° in screw-capped bottles without preservative. Serum was inactivated immediately before use by heating at 56° for 30 min.

EXPERIMENTAL

Isolation of Cytopathogenic Viruses from Adenoids

Roller tube cultures of adenoid tissue were observed for an average of four weeks. At various times after the first week cells which had spread out in flat epithelial-like sheets were seen to become rounded, highly refractile and tending to form clumps. When all the cells had rounded up, they and the fluids were harvested, homogenised together, and stored at −26° for further study. Frequently fluid removed from cultures beginning to show this degenerated appearance was also stored for further study. Permanent stained preparations were made of some cultures in various stages of degeneration. Bacterial sterility tests were made. The stored fluids were sub-inoculated into cultures of HeLa cells and passaged at least twice. If the same typical cytopathogenic change was seen, together with a rapid pH change in the medium towards the acid side, isolation of a virus was considered to have been made.

Altogether 22 adenoids were obtained, and satisfactory cultures were made of 18 of these. Six viruses have been recovered and are shown below to possess the

properties of the APC virus group. Many of the properties to be described were studied using one or both of the first two viruses to be isolated (HA12 and 13). Pools of each strain were made by inoculation of cultures of HeLa cells, and harvesting cells and fluid when the cells fell off the glass. Pools were stored at −26°.

Range of pathogenicity

Volumes of 0·1 ml. of a virus pool were inoculated into cultures of various tissues. The results obtained are summarised in Table I and the pathological

TABLE I.—*Some Properties of Viruses Recovered from Tissue Cultures of Adenoids in Sheffield*, 1954–55.

Property tested.	Tissue or animal inoculated.	HA4.	HA12.	HA13.	HA15.	HA16.	HA17.
Production of degenerative changes in tissue cultures	HeLa (with acid production)	+	+	+	+	+	+
	Human embryo lung	+	N.T.	+	+	+	N.T.
	Human embryo kidney	+	+	+	+	+	+
	Human embryo fibroblasts	N.T.	N.T.	+ (delayed)	N.T.	N.T.	N.T.
	Human kidney*	+	+	+	+	+	+
	Monkey kidney	+†	+	+	+	+	+
	Rabbit kidney	+	+	+	+	+	+
	Chick embryo lung	−	−	−	−	−	−
Type-specific neutralisation	Rabbit kidney	+ (Type 2)	+ (Type 2)	+ (Type 5)	+ (Type 5)	+ (Type 1)	+ (Type 2)

* Viruses HA4, HA15 and HA17 were tested in human kidney cells in continuous culture (Chang, 1954).
† This strain caused complete destruction of monkey kidney cells.
N.T. = Not tested.

changes are illustrated in the Plate. All the human tissues showed degenerative changes after inoculation, and the epithelial cells seemed to be uniformly susceptible. The fibroblasts tested in pure culture degenerated much later than epithelial cells. In the human lung cultures the tubes contained both epithelium-like cells and fibroblast-like cells. It can be seen in the Plate that the latter were intact when marked changes had taken place in the former. Later the fibroblast-like cells also degenerated. The changes seen in monkey kidney and rabbit kidney cells were similar. However, after the first few days the degenerative process seemed to remain almost static, while in the human epithelial cells and fibroblasts the process always led finally to every cell rounding up and falling from the glass. Complement-fixing antigen was produced in infected rabbit kidney cultures in titres up to 16, but little infectious virus appeared.

In preparations stained with haematoxylin and eosin it could be seen that striking nuclear changes accompanied the " rounding up " of the cells. The nuclear material became condensed and uniform or broke into small fragments. The cytoplasm at times seemed more strongly eosinophilic than normal. Some of the abnormally dark nuclei were rather large, but no definite inclusion bodies were seen although others have reported masses of intranuclear particles (Kjellén, Lagermalm, Svedmyr and Thorsson, 1955) in cells infected with these viruses.

Two strains (HA12 and HA13) were tested by inoculation into various animals. Five-week-old mice were inoculated by the intracerebral and intranasal route, and suckling mice and suckling hamsters by the intracerebral and intraperitoneal routes. All animals remained quite healthy for four weeks. No attempt was made to recover virus from the animals or to carry out blind passages in them.

Fluids from degenerating cultures from all 18 adenoids investigated were inoculated by the amniotic route into 13-day-old embryonated hen's eggs. Amniotic and allantoic fluids were harvested after 48 hr. incubation at 35°, and tested for haemagglutination with fowl cells and human group O cells, at room temperature and at 4°. A second and sometimes a third passage was made by the amniotic route. No positive haemagglutination results were found which were reproducible on passage, *i.e.*, viruses of the influenza group were apparently not present.

Physico-chemical properties

Five strains were tested for ether resistance. Samples of undiluted fluids containing each of the five strains were mixed with equal volumes of anaesthetic ether, and left overnight at 4°. Next morning most of the ether layer in each tube was removed by pipetting, and the remainder was allowed to evaporate in the 37° water-bath. The fluids were tested in cultures of HeLa cells for the presence of virus. Virus-containing fluids untreated with ether were also inoculated into other HeLa cell cultures as controls. All five agents tested were completely resistant to ether treatment. No difference was observed between cultures inoculated with treated and untreated fluids.

Six strains were tested for heat stability. Samples of undiluted fluids were held in a 56° water-bath for 30 min. The fluids were then tested in cultures of rabbit kidney cells for the presence of virus. Unheated fluids containing each agent were also inoculated into similar cultures as controls. No cytopathogenic changes were observed in the test cultures, while full cytopathogenic changes occurred in controls.

Three strains tested retained the ability to produce cytopathogenic changes in cultures of human cells after passage through bacteria-retaining sintered glass filters (Pyrex 5/3) and two others after passage through collodion membranes (A.P.D. 650–700 mμ.).

All of six strains tested were apparently unaffected by the penicillin and streptomycin incorporated in the culture media as described earlier and must therefore be to this extent resistant to these antibiotics.

Serological properties

Six strains were tested by neutralisation tests in rabbit kidney against type-specific immune sera prepared against types 1–6 of APC virus. These sera were kindly supplied by Dr. H. G. Pereira. Each agent was found to be neutralised by one, and only one, of the six type-specific antisera used.

Two viruses (HA12 and HA13) were tested for the possession of complement-fixing antigen, using a human immune serum, kindly supplied by Dr. M. R. Hilleman, known to contain complement-fixing antibody against the APC viruses. One was identified as type 2 by neutralisation test, and the other as type 5. Both gave positive complement-fixing reactions. Human sera collected locally and shown to contain complement-fixing antibody to high titres against the original strains were used in testing four more strains. These belonged to type 2 (2), type 5 (1),

and type 1 (1) by neutralisation tests ; they were all found to produce complement-fixing antigen. The titres of the human sera were about the same irrespective of the strain used to prepare the antigen.

It was concluded that the six cytopathogenic agents isolated all possessed properties considered by Huebner *et al.* (1954) to be those of viruses of the APC group.

Serological Investigations

Evaluation of serological tests

A pool of three specimens of human serum was titrated for neutralising antibody against the APC viruses types 1–6, twice in rabbit kidney cultures and once in HeLa cell cultures. In each case the serum was titrated in fourfold dilutions. Antibody against each strain was detected by both procedures as is shown in Table II but the titres obtained did not correspond exactly, which may be a result of the differing amounts of virus used in the two tests. HeLa cell cultures

TABLE II.—*Comparison of Results of Neutralisation Tests on Pooled Human Serum in Rabbit Kidney and HeLa Cell Cultures*

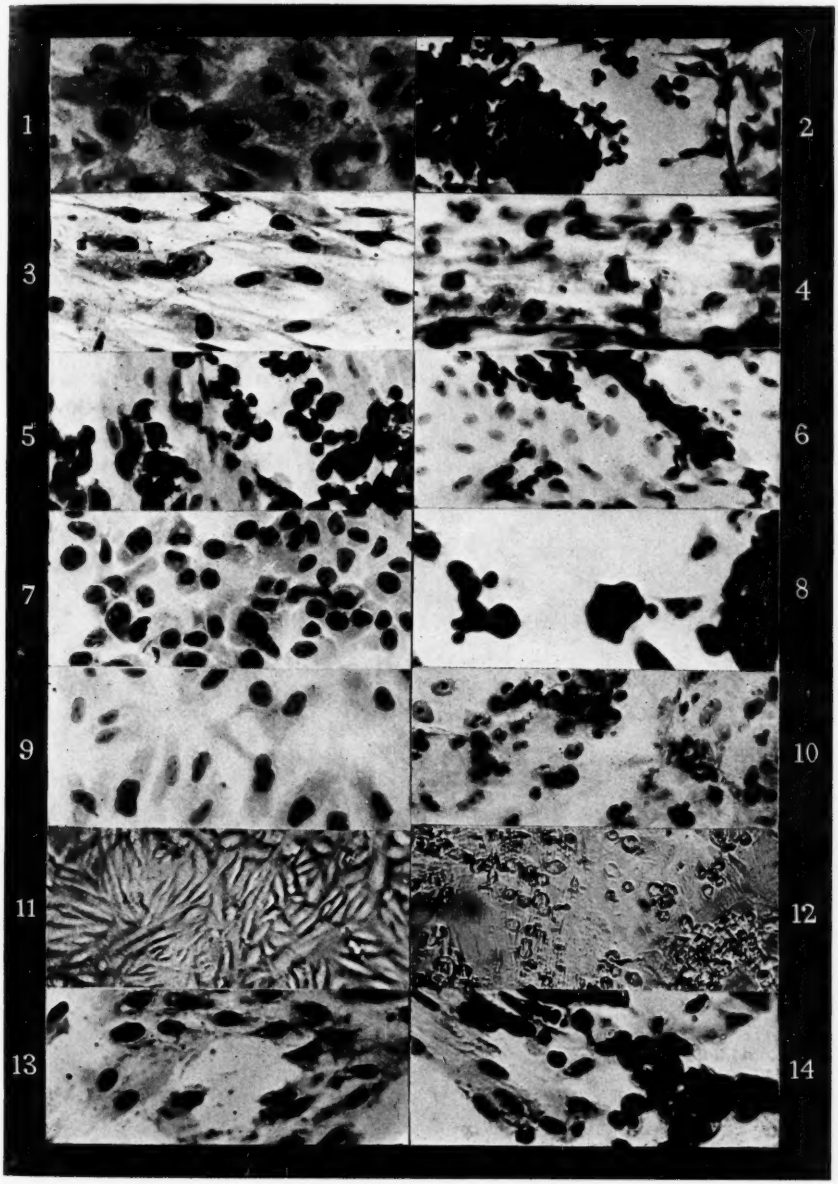
Virus type.	Dilution of virus* used in		Serum neutralisation titre using		
			Rabbit kidney.		
	Rabbit kidney.	HeLa.	Exp. I.	Exp. II.	HeLa.
1	1/10	1/50	16	16	32
2	1/10	1/300	64	64	>256
3	1/5	1/1000	32	16	128
4	1/5	1/100	512	8	16
5	1/10	1/50	32	32	32
6	1/10	1/300	16	16	16

* The same virus pools were used in all experiments.

EXPLANATION OF PLATE

Appearance of cells growing in tissue culture and infected with virus strains isolated from adenoids removed in Sheffield. Stained preparations were fixed in Bouin's solution and stained with Mayer's haemalum and eosin and photographed at a magnification of about × 200. The unstained preparations were photographed with conventional lighting at a magnification of × 100. The inoculum in each case was a virus pool obtained from infected HeLa cells.

1.—Intact sheet of epithelium-like cells from human adenoid.
2.—Sheet of epithelium-like cells from human adenoid showing degeneration. Note the presence of normal fibroblasts. A cytopathogenic agent was recovered from the culture fluids.
3.—Normal human embryo fibroblasts.
4.—Human embryo fibroblasts infected with HA13, 14 days after inoculation.
5.—Human embryo lung infected with HA4. Surviving normal fibroblasts, 2 days after inoculation. Note round or distorted dense nuclei, and some cells with large dense nuclei.
6.—Human embryo kidney infected with HA17. Normal and abnormal epithelial cells 3 days after inoculation.
7.—Uninfected human epidermoid carcinoma (strain HeLa).
8.—Epidermoid carcinoma (strain HeLa) infected with HA13.
9.—Normal monkey kidney cells.
10.—Monkey kidney cells infected with HA13. Normal and abnormal cells.
11.—Normal rabbit kidney. Unstained.
12.—Rabbit kidney infected with HA13, 3 days after inoculation.
13.—Normal rabbit kidney. Stained.
14.—Rabbit kidney infected with APC type 2 (from Dr. H. G. Pereira) 6 days after inoculation.



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gave higher titres on the whole, and with the exception of type 4, the results obtained in rabbit kidney cultures were reproducible.

In order to compare the results of complement fixation tests on plates with tests carried out in tubes, sera positive against influenza A or B were titrated on plates and by a standard procedure in tubes. The results agreed within the limits of experimental error.

In order to check the specificity of the influenza C and APC antigens several human sera positive against these viruses separately or in combination were set up with control antigens against various chicken tissues and various tissue culture fluids. No evidence was obtained of the presence of cross-reacting or non-specific components in the antigens used.

In tests on 40 sera tested by both haemagglutination-inhibition and complement fixation tests against influenza C there was reasonable correlation between the results obtained by the two methods.

Titrations of paired sera from cases of respiratory disease

Paired sera were collected from cases of acute disease ranging from colds to mild pneumonia admitted to various Army hospitals between 1952 and 1955. The sera were titrated by complement fixation tests against influenza A, B and C and APC viruses.* Paired sera from 67 cases of bronchitis and pneumonia mostly over the age of fifty admitted to the City General Hospital, Sheffield, between October, 1953, and March, 1954, were also titrated. A fourfold or greater rise in antibody level (between the acute and the convalescent phase serum specimens) was regarded as " positive " for the antigen concerned and in nearly every case the titre for APC or influenza C was confirmed by at least one repetition of the test.

The results are summarised in Table III : examples of the titres found in positive sera have been given in an earlier publication (Zaiman *et al.*, 1955). The

* Except for a group of sera from Chester collected after November, 1954, which were not titrated against influenza C and APC when they had been found to be positive against influenza B.

TABLE III.—*Results of Complement Fixation Tests on Paired Sera from* 302 *Adults Suffering from Acute Febrile Illnesses Involving the Respiratory Tract**

Place.	Period.	Number tested.	Positive results* for—					
			Influenza.					
			A.	B.	C.	APC.	A and APC.	B and APC.
Catterick Army Camp	January and February, 1952	9	0/9	0/9	0/9	2/9†	0/9	0/9
City General Hospital, Sheffield	November, 1953–March, 1954	67	0/67	0/67	0/67‡	3/67	0/67	0/67
Chester Army Camp	May, 1954–March, 1955	150§	4/150	56/150	1/84	14/84		
Retford Army Camp	May, 1954–June, 1955	76	0/70	15/70‖	4/74	5/73	1/64	1/63
	Totals	302	4/296	71/296	5/234	24/233	1/140	1/139

* "Positive result" means a confirmed × 4 or greater rise in C.F. antibody titre against the virus.
† The numerator is the number positive and the denominator is the number tested.
‡ 4 cases with very high titres (320–1024).
§ 53 positive for influenza B and A from March, 1954–March, 1955, not tested further.
‖ One had anti-influenza A antibodies 28/80.

important points are that over the whole period studied, and in each geographical area sampled, and in both soldiers and civilians there is definite evidence that antibody responses against APC viruses occurred during acute infections of the respiratory tract. What part the virus played in causing these infections is not indicated by these data, and the epidemiological and clinical aspects of the results will be presented elsewhere.

Table IV shows the results of complement fixation and neutralisation tests carried out on paired sera from 6 soldiers. The sera were titrated for neutralising antibodies after the complement fixation test had shown a positive result and if sufficient serum was available.

It was hoped to confirm the rise in antibodies and also to learn which serological types had or had not caused these infections. Three cases showed neutralising antibody rises against one or other of the APC viruses. Three cases occurring in the Chester outbreak showed no neutralising antibody rises against the viruses employed and perhaps this outbreak was associated with a different serological type from the six varieties included in the test. It is of interest that there were no antibody rises against the type 4 viruses which have been isolated in cases of acute respiratory disease in American soldiers.

TABLE IV.—*Antibody Titrations on Sera from Cases Positive in APC Complement Fixation Tests*

Patient.	Locality.	Year.	C.F. test with influenza.* A.	B.	C.	APC.	Neutralisation test with APC.† 1.	2.	3.	4.	5.	6.
Y—	Catterick	1952	<4	<4	320‡	6	<16	<16	<16	128	<16	<16
			<4	<4	160‡	48	160	40	40	40	<20	<20
B—	Retford	1954	—	—	<4	<4	32	<8	<8	<8	<8	<8
			8	8	<4	24	128	8	128	8	<8	<8
G—	Retford	1954	<4	<4	<4	<4	<8	<8	32	<8	<8	<8
			<4	<4	<4	16	<8	8	>256	<8	8	<8
M—	Chester	1955	—	—	16	16	<16	64	<16	<16	<16	16
			<8	<8	32	128	16	64	16	<8	<8	8
H—	Chester	1955	—	—	16	8	<16	<16	<16	<16	<16	<16
			8	8	16	64	<16	<16	<64	<16	<16	<16
H—	Chester	1955	—	—	10	4	8	16	<8	<8	<8	16
			<8	<8	12	16	16	64	<16	<8	8	<8

* The upper figure is for the acute and the lower for the convalescent titres. Unequivocal rises are enclosed in heavy lines.

† Partial protection end-points in rabbit kidney cultures.

‡ Haemagglutination inhibition titre.

Survey of sera collected from various groups

Complement fixation titrations against APC viruses and influenza C were carried out on the individual sera of various population groups and acute phase sera from the patients described in the previous section who were " negative " for APC infection. In all groups antibody was detected against both viruses. The results are summarised in Fig. 1. It will be noted that there appears to be more antibody in the sera of the soldiers and factory workers than the medical students, and that in the former some of the titres fall in the range found in convalescent sera. The result might be due to many factors, such as differences in environment or times of collection. In Fig. 1 it is seen that there were higher titres in the soldiers

against both viruses in sera collected in winter than in sera collected in summer. Titrations of paired sera showed that there was an increased incidence of APC virus infections in the military groups during the early months of 1955 and there may have been similar differences in the experience of the students and factory workers in the period preceding the collection of sera. There were a few extremely high titres against influenza C and APC in the group from the City General Hospital, Sheffield, suggesting that some of these had had recent severe infections with these viruses which may have contributed to their illness.

Serum from child 13 was titrated against HA13, the virus isolated from its adenoids. The serum neutralised virus at a dilution of 1/64, but not at 1/256. Serum from child 12, from whom a type 2 virus was recovered, was titrated against type 2 virus. The serum neutralised at a dilution of 1/64. Neither of these sera had detectable complement-fixing antibodies at a dilution of 1/4.

Titrations of pooled human sera

Sera collected in 1953 in the Sheffield area were titrated in pools representing groups of 50 persons covering an age range of 2 years.* There were no pools available in a few age groups, namely 2–5, 40–43, 54–57 yr.

The serum titres of the pools within a particular decade were averaged by taking the arithmetic mean counting a titre of less than 4 as zero. The results (Fig. 2) show that complement-fixing antibody is found mainly in sera of persons under the age of 30 years. There is a definite contrast with the control results using influenza C virus in which it is seen that antibody is found in pools from both old and young persons.

Neutralising antibody titrations against 6 types of APC virus have been carried out on similar pools, each covering a range of about 10 years, and deriving from about 250 individuals. The results show that neutralising antibodies are found against all types of virus tested and are present at various levels in different age groups.

DISCUSSION.

The results of our attempts to isolate viruses from human adenoids confirm the work of Rowe *et al.* (1953) and we have been able to identify our agents completely as members of the APC group as defined by Huebner *et al.* (1954). The serological types of the strains isolated in Sheffield agree well with those which Huebner found in viruses obtained from adenoids in Washington; our serological findings also seem to agree well with American studies both as to the prevalence of C.F. antibodies and the age at which they are found (Huebner *et al.*, 1954; Hilleman, Werner, Dascomb and Butler, 1955). We also agree with American workers in finding cases of disease apparently associated with infection by these viruses in soldiers during the winter. However, we did not find the very high incidence demonstrated by Hilleman although the Service population studied included recruits. We intend to investigate further the results suggesting that APC virus infection may cause some cases of acute bronchitis in elderly civilians. Comparison of the results of C.F. and neutralisation tests in paired sera showing a rise of antibody titre confirms that the C.F. test is of broad specificity and convenient for diagnosis.

* The results of influenza antibody titrations on these serum pools have been published by Davenport, Hennessy, Stuart-Harris and Francis (1955).

The only parallel results to ours on C.F. antibody levels against influenza C virus suggest that the frequency of C.F. antibodies in Britain and Hungary is similar to that which we found (Andrews and McDonald, 1955; Farkas and Dömök, 1954). The fact that antibodies against influenza C are found in all age groups except young children has been mentioned by Hilleman, Werner and Gauld (1953).

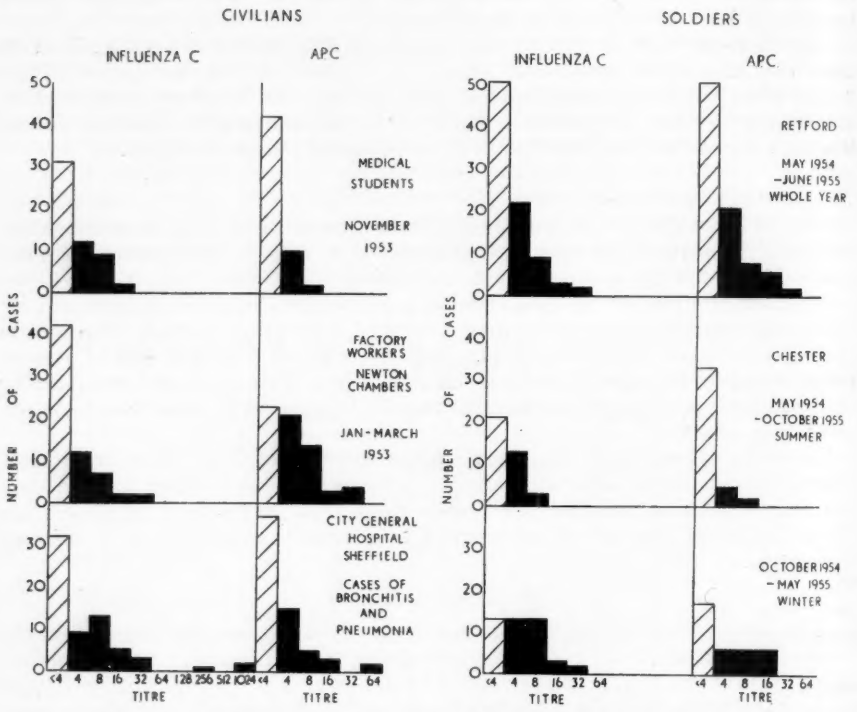

FIG. 1.—Frequency of complement-fixing antibody titres in sera from various populations.

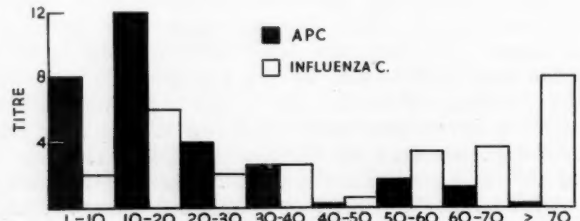

FIG. 2.—Mean complement-fixing antibody titres in pools of sera from normal persons of different age groups collected in the Sheffield area.

The results of inoculating APC viruses into various tissue cultures have raised a number of questions. One of these is whether rabbit kidney cultures might not be more widely used for studies of other viruses (Barski, Lamy and Lépine, 1955) including agents which are not readily propagated in them. The fact that APC viruses are found in so many adenoids removed at operation and that they persist in spite of the presence in the serum of neutralising antibody suggests that these viruses could be connected with chronic affections of the upper respiratory tract. In view of the name given to these viruses it is well to remember that the extent of their pathogenicity for man is by no means fully defined. This suggestion is also encouraged by the work of Kjellén referred to earlier.

Although it has been shown that human influenza virus can be propagated in human and monkey kidney cultures (Mogabgab, Green and Dierkhising, 1954; Mogabgab, Simpson, Dierkhising and Green, 1955) we were unable to isolate influenza viruses from 18 adenoids, from 6 of which we recovered APC viruses. This fits the current view that there is little evidence for long-continued latent infection with influenza virus in man. The fact that we isolated types 1, 2 and 5 from adenoids confirms the findings of Huebner *et al.* (1954) and confirms that these strains are usually found in adenoids and tonsils, while types 3 and 4 are usually found in association with epidemic disease.

In laboratory studies on a variety of clinical syndromes it is now desirable to test sera for antibodies against a large and increasing number of viruses, many of which must be propagated in tissue culture or yield relatively low titre antigens. Our experience in this and other investigations leads us to believe that for all the conditions just mentioned a complement fixation test on plastic plates is a very valuable method suitable for use in routine diagnosis.

SUMMARY

Six viruses have been recovered from tissue cultures of adenoids removed in Sheffield. The viruses cause degeneration of a wide range of cells; they are ether-resistant and react specifically in neutralisation and complement fixation tests with antisera against viruses of the APC group.

Of 233 paired sera from cases of acute respiratory disease, 24 showed a rise in complement-fixing antibody titre against these viruses. Complement fixing and neutralising antibodies against the APC viruses are widely distributed in sera collected in the North of England.

Complement fixation tests were carried out on perspex sheets and neutralisation tests in tissue cultures of rabbit kidney cells.

This work was carried out with the aid of a grant from the Medical Research Council. We wish to acknowledge the help of Lt.-Col. McKelvey, Lt.-Col. Cook, Captain Dennis and Captain Pate of the R.A.M.C. and also Dr. F. J. Flint for supplying clinical data and sera from their patients. We also wish to thank Dr. J. C. Whitehead for carrying out some complement fixation tests against influenza A and B, and Mr. J. D. Gray, F.R.C.S., Professor Scott-Russell, F.R.C.O.G., Mr. J. C. Anderson, F.R.C.S. and Mr. R. B. Zachary, F.R.C.S. for supplying various human tissues.

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The work was carried out under the direction of Professor C. H. Stuart-Harris and Professor C. P. Beattie.

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INHIBITION OF INFLAMMATORY RESPONSE OF SKIN TO *STAPHYLOCOCCUS AUREUS* BY HIGH POLYMER LEVAN

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PREVIOUS studies have shown that high polymer levan and dextran lower the resistance of the mouse to *Salmonella typhi* inoculated into the peritoneal cavity (Olitzki, Shelubsky and Hestrin, 1946; Hestrin, Shilo, Feingold and Wolman, 1954). The experimental evidence suggested that transfer of antibacterial agents from the blood to the locus of infection was diminished by the polysaccharides. It has been stated in a preliminary communication that high polymer levan prevents the local manifestations of acute inflammation (Shilo, Wolman and Hestrin, 1954). Davies, Shilo and Hestrin (1955) have shown that passage of trypan-blue and antibody protein across the endothelium at the site of inflammation is blocked by levan and other high polymer saccharides. The present study describes the effect of high polymer levan on the response of skin to *Staphylococcus aureus*.

MATERIALS AND METHODS

Polysaccharide.—The solution of native high polymer levan of *Aerobacter levanicum* was prepared as previously described (Hestrin, Shilo and Feingold, 1954).

The polysaccharide was injected intravenously, intraperitoneally or intracardially as a 3 per cent solution in saline after heating to 80° for 15 min. and centrifuging for 10 min. at 2000 r.p.m. Unless specified otherwise, rabbits were given a single dose of 100 mg. polysaccharide/kg. body wt. Guinea-pigs received 300 mg. per kg.

Bacteria.—The strain of *Staph. aureus* used was isolated from a case of sheep mastitis and kindly supplied by Dr. T. Dishon. Bacteria from 24 hr. slants were washed with saline and made up in suspension to contain 10^6–10^7 bacteria per ml. Doses of 0·1 ml. were injected intradermally.

Animals.—Adult rabbits weighing 1·5–2 kg., and guinea-pigs weighing 300–400 g. were used. Study of gross lesions was made on a group of 15 levanised rabbits and 6 levanised guinea-pigs. The microscopical studies were made on the skin of 4 rabbits and 4 guinea-pigs given levan; in both series an equal number of animals served as non-levanised controls.

The skin of the abdomen of the animals was depilated with BaS before the inoculation of the bacteria.

The appearance and spread of the lesions were followed visually. The animals were eventually sacrificed and the areas of lesion were excised and fixed in a 10 per cent formalin solution. Paraffin-embedded blocks were cut at 8μ and stained with haematoxylin and eosin by Weigert's Gram method. Some sections were stained by both methods.

RESULTS

Non-levanised rabbits and guinea-pigs inoculated intracutaneously with bacteria reacted with the formation of well delineated abscesses at the sites of inoculation. Macroscopically congestion became visible after about 2 hr., and easily recognisable

abscesses formed in 10–12 hr. Microscopically diapedesis of leucocytes was first visible after 15–30 min.; by 6–8 hr. there was a definite abscess formation with the presence of purulent material in the centre.

In rabbits which were given levan up to 8 hr. before the inoculation with bacteria, macroscopic signs of inflammatory response were markedly delayed. For about 4 hr. the area showed no gross signs of damage, except for occasional oedema. At 4–6 hr. a region of pallor was noted around the inoculated site; subsequently frank dermonecrosis appeared and spread rapidly during the first 12 hr. By 24 hr. purple or dark red patches often occurred in the boundary of the area of dermonecrosis.

Microscopic findings on lesions of different stages of development in levanised animals (lesions aged 15 min., 30 min. and 1, 2, 4, 6 and 8 hr. respectively, levan given prior to the first injection of bacteria) may be summarised as follows. The number of bacteria in the sections seemed to increase progressively. The bacteria spread to the walls of the blood vessels. The blood vessels were congested, but no diapedesis was observed. The inflammatory oedema, if present, was minimal. During the first 8 hr., except for the presence of multiple bacteria and of congestion, the tissue appeared almost undamaged. There was some loss of fibrillar structure in the dermis which appeared more homogeneous and slightly more basophilic than the surrounding tissue. In some instances there was evidence of a change in the subcutaneous fat which contained some stainable material within the fat vacuoles and appeared granular and more basophilic. At later stages the epidermis became necrotic, and there was evidence of necrosis in the dermis, fat and underlying muscle. Lesions which were left to develop for more than 24 hr. showed a slight leucocytic reaction around the necrotic area. Similar results were obtained in animals which were given levan intraperitoneally, provided the injection was given 2 hr. before the staphylococci.

In view of the known leucopenic effects of many high polymers it should be noted that numerous neutrophilic granulocytes were found within capillaries around the sites injected with staphylococci.

Some rabbits and guinea-pigs were given levan 5 hr. after the inoculation of bacteria. In these animals the inflammatory response was interrupted by the levan. Necrosis developed which was characterised by extreme congestion and haemorrhages. The necrotic area appeared black. Microscopically there was evidence of extensive necrosis accompanied by an unusually low concentration of neutrophilic leucocytes.

Levan in a dose of 100 mg./kg. regularly produced in the rabbit the grey-yellow dermonecrotic lesion with well defined boundary which has been described. Threshold doses of levan (10–50 mg./kg., the value varying with levan batch) produced a diffuse haemorrhagic dermonecrotic lesion. The order of magnitude of the levan dose producing dermonecrotic response towards staphylococcus was the same as that which blocks extravasation of trypan blue and antibody protein at a site of inflammation (Davies *et al.*, 1955).

DISCUSSION

These experiments show that high polymer levan inhibits the exudative phenomena of acute inflammation. In the levanised animal the bacteria multiply seemingly unhindered by leucocytes and antibodies.

Miles and Niven (1950) have shown that shocking agents inhibit acute inflammation and enhance staphylococcus infections. However, levan does not produce shock and hence must be acting by a different mechanism. Cortisone, too, is known to inhibit acute inflammatory response and to produce lesions similar to those described above (Rebuck and Mellinger, 1953 ; Robinson and Smith, 1953). It is not clear whether this effect of levan and cortisone is based on the same or on different mechanisms.

SUMMARY

High polymer levan administered systematically inhibits the acute inflammatory reaction of rabbit and guinea-pig skin to staphylococcus and causes spreading dermonecrosis in the infected area.

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